GENETIC AND BIOCHEMICAL ANALYSIS OF MATERIALS FROM A MEDIEVAL POPULATION FROM YNYS MÔN, NORTH WALES

By

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A dissertation submitted in complete fulfilment of the requirements for the degree of

PhD

University of Central Lancashire

Declaration

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

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ABSTRACT

GENETIC ANALYSIS OF A MEDIEVAL POPULATION FROM YNYS MÔN, NORTH WALES

By Ashley Matchett

The archaeological excavation of the early medieval site at Towyn-Y-Capel on the island of Anglesey (Ynys Môn) in North Wales, UK, provided the opportunity to study a large population (122 skeletons) at a site that was in use over a period of up to 550 years (650 -1200 AD). Samples of skeletal materials for this study were taken directly from the site itself .The osteological condition of skeletal material was variable across the site. In general, the upper burials in particular were in the poorest condition, and were mainly fragmented and dispersed due to the ongoing site erosion and diagenetic processes. Conversely, lower "cist" burials were in far better condition.

The assessment of skeletal sample condition was used to select materials chosen for genetic analysis, and 44% (54) of the skeletal population were selected for analysis of appropriate samples of tooth and bone. The gross morphology of samples was assessed and 87% of bones and teeth were considered to be in good or fair condition, according to the gross preservation index (GPI) used, while only 2% of bones and no teeth were considered to be in excellent condition. In addition to GPI, a novel technique called Qualitative Light Fluoresence (QLF), based on autofluoresence, was used to ascertain the surface condition of the teeth. Compared to the fluorescence of modern enamel, there was a net loss of 21.8% fluorescence, although the degree of fluorescence from one sample to another varied (with a standard deviation from the mean of 24.973). Histological sections taken from non-human bone finds from the site generally varied less than that indicated by the gross morphology, showing good to excellent histological preservation.

Further to gross and histological morphology, ten skeletal samples were selected for detailed investigations, and were analysed for amino acid racemisation and amino acid composition. All samples tested had D/L enatomer Aspartic acid ratio less than 0.1, although 50% of the samples had D/L enatiomer Aspartic acid ratio over 0.08, which indicated that the recovery of aDNA from these skeletal samples was feasible, although the biological condition of the teeth was fairly degraded. The inorganic element profile of the same ten samples showed no discernable anomalies, either due to diet or diagenesis. To consolidate genographic research, strontium isotope analysis was performed and, from the small population subset, three anomalous ratios were found.

Two of these were high (Skeletons 33 and 60), indicating that these individuals had spent their childhoods in areas with high strontium ratios, representative of precambrian rock types, possibly older than those of the Holyhead Rock group, such as in Northern Scotland or Norway. The skeletal samples yielding the lowest strontium ratio (Skeleton 52) are of compelling interest, since the ratio is indicative of upbringing in only one place in the North Atlantic, namely Iceland.

In this study, DNA recovery was performed on teeth and bones from the site, after extensive decalcification of samples, and also extraction and optimisation trials. Amplification of DNA extracted from teeth samples was generally more successful than for bone samples. A random amplification based polymorphic (RAPD) DNA technique was utilised to "fingerprint" human and animal samples with limited success. Contamination and template variation are likely causes for the lack of success. Amplification using several primers specific for human HV1 & 2 mtDNA targets was also met with limited success. The results show that 14.8% of the skeletal teeth samples were amplified, and these were not commonly reproducible. DNA spiking trials demonstrated that some of the samples were affected by inhibition. Independent confirmation of 9 of 10 successful samples was attained by sequencing, and although sequences were highly degraded, an attempt was made at determining the haplogroups from the sequenced HV1 haplotypes based on likelihood. Generally, the site showed a high predominance of Haplotype K (5) followed by H (2) and U (2) haplogroup profiles.

Keywords: - Ancient DNA, ancient population, gross morphology, histology, quantitative light fluorescence, mitochondrial DNA, random amplified polymorphic DNA, amino acid racemisation, strontium isotopes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF TERMS AND ACRONYMS

A	adenine
AAR	amino acid racemization
AAS	atomic absorption spectrometry
aDNA	ancient DNA
AP-PCR	arbitrarily primed PCR
Ala	alanine
Asp	aspartate
Betaine	N,N,N -trimethylglycine
BP	years before present
bp	base pair
BSA	bovine serum albumen
C	cytosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuracil triphosphate
EDTA	ethylenediaminetetraacetic acid
*f*g	femtogram
G	guanidine
GAT	Gwynedd Archaeological Trust
GC-MS	gas chromatography-mass spectrometry
Glu	glutamine
Gly	glycine
GPI	gross preservation index
Hg	haplogroup
HPI	histological preservation index
HPLC	high performance liquid chromatography
Ht	haplotype

HV/HVR	hypervariable region
ICP-MS	inductively coupled plasma-mass spectrometry
LA-ICP-MS	laser ablation-ICP-MS
LCN DNA	low copy number DNA
mtDNA	mitochondrial DNA
MC-ICP-MS	multiple collector-ICP-MS
NCBI	national center for biotechnology information
ηg	nanogram
O.D.	ordanance datum
PCR	polymerase chain reaction
pg	picogram
ppm	parts per million
PTB	N-phenacyl thiazolium bromide
QLF	quantitative light fluorescence
RAPD	random amplified polymorphic DNA
rCRS	revised Cambridge reference sequence
RNase	Ribonuclease
ROS	reactive oxygen species
RP-HPLC	reverse phase-HPLC
RT-PCR	real time PCR
SDS	sodium dodecyl sulphate
Ser	serine
Sk	skeleton
SNP	single nucleotide polymorphism
STR	short tandem repeat
T	thymidine
Taq	*taq* DNA polymerase
TBE	tris Borate EDTA
Tm	melting temperature
U-DNA	uracil-DNA
µg	microgram
UNG	uracil N-glycosylase
UV	ultra-violet
UV/vis	UV/visible light spectrum

CHAPTER 1

INTRODUCTION TO TOWYN-Y-CAPEL, HARD TISSUES AND ANCIENT BIOMOLECULES

1 INTRODUCTION TO TOWYN-Y-CAPEL, HARD TISSUES AND ANCIENT BIOMOLECULES

TOWYN-Y-CAPEL

Towyn-Y-Capel or Capel St. Ffraid (Latitude 53^{o}16'45.4''N Longitude 4^{o}37'0.8'' W), is the site of a former chapel and cemetery. This site is believed to have been first used as a burial ground in the 7th century AD, and appears to have been in constant use until the 17th century. The chapel, constructed in the 12th century AD, and a significant part of the burial mound was destroyed during a fierce storm in 1913 leaving only a segment of the original mound close to the seashore. The site has been subjected to further coastal erosion since then and this led to the need to excavate and recover the remains before the mound is completely destroyed.

The site is located on the West Coast of Holy Island, Anglesey, in the Parish of Holyhead (Figure 1.1). It lies just above the high water mark in the centre of an indented sandy bay now called Trearddur, but formerly called Saint Bride's (or St Ffraid's) bay, after the dedication of the chapel to St Ffraid (Figure 1.2). The land is low lying in the immediate vicinity of the mound, although there are rock outcrops. To the East, there is a tidal inlet which stops only 450 m east of the chapel site and which almost cuts Holy Island into two parts. It has been suggested that this inlet was once the estuary of the river Alaw and that the bay of Trearddur would have contained the mouth of the river, similar to Ffraw at Aberffraw (Greenly, 1919). On the beach below the mound, the inter-tidal area is interspersed by a layer of peat, tree stumps and the petrified remains of trees. Similar remains further round the coast at Llanddwyn and was carbon dated to 6295 +/- 90 BP (years Before Present) (Williams, 1996).

Prior to excavation, the site was visible as a low sand dune lying just above the high water mark, and separated from the beach by a promenade. The mound measured some 40 m North to South and 20 m East to West. The top lay 4.42 m above the adjacent promenade, and 6.05 m above the beach. The promenade lies at about 4.5 m OD (Ordnance datum {height above sea level}), so the top of the mound was roughly 9 m OD. The site is crossed from West to East by a stone wall, which was built in the early years of the 20th century AD. A number of stone slabs, some on edge, were visible within the turf on the mound. Some of these stone slabs are thought to be the remains of cist graves.

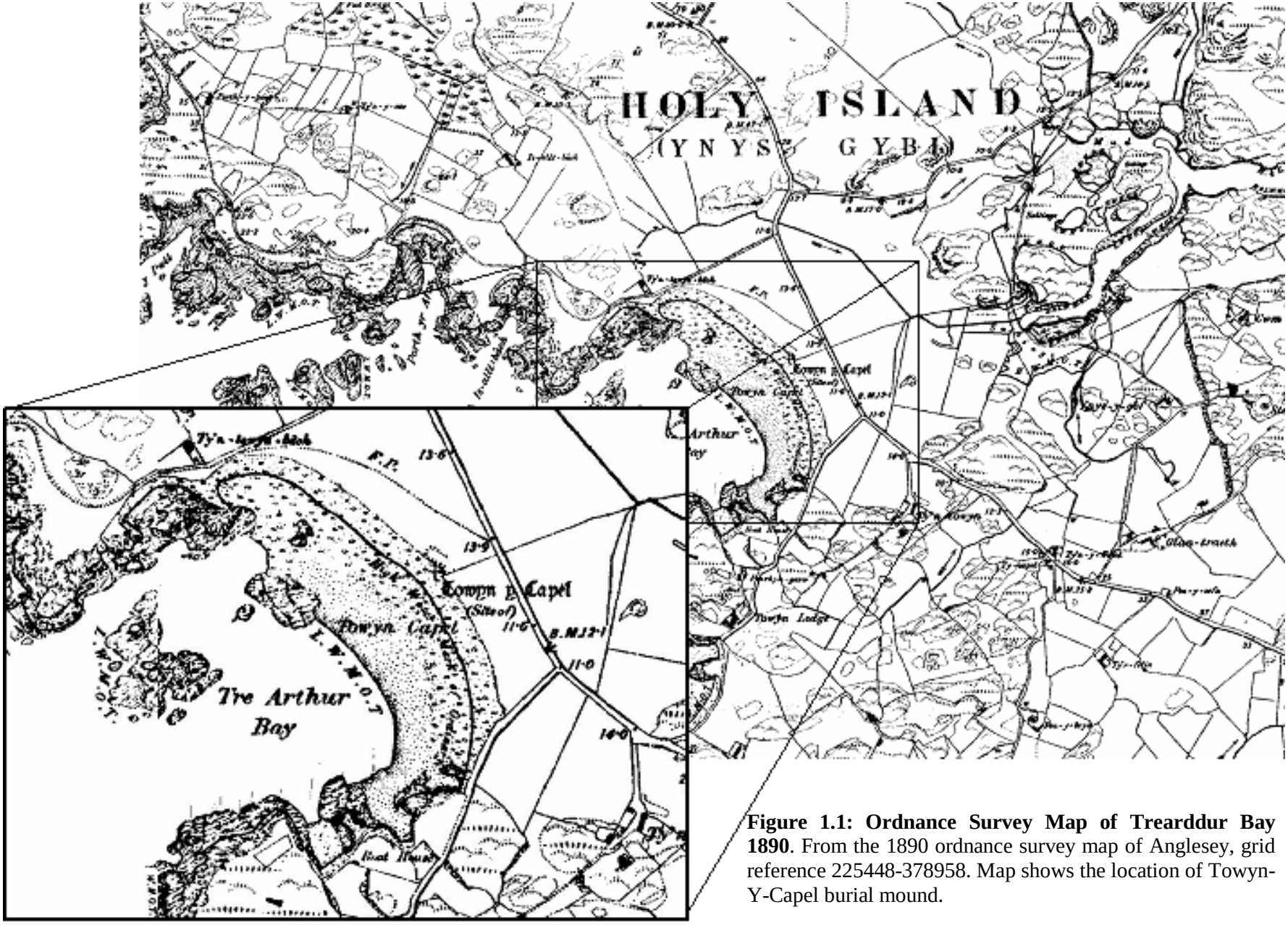


Figure 1.1: Ordnance Survey Map of Trearddur Bay 1890. From the 1890 ordnance survey map of Anglesey, grid reference 225448-378958. Map shows the location of Towyn-Y-Capel burial mound.

Figure 1.2: Aerial Photograph of Trearddur Bay.
Overhead photo of Trearddur beach shows the modern day location of the Towyn-Y-Capel site. Photograph courtesy of GAT.

The site provided a unique opportunity to study a relatively isolated community over a prolonged chronological period. Morphological analysis of the buried population, in terms of age, sex and family group analysis, coupled with the chronology of the site, was carried out to provide data on the inhabitants of this area during the middle to late medieval period. The biomolecular analysis was intended to establish individual, group and local population genealogy to compliment the anthropological and archaeological data.

1.1 Archaeological Investigation into Towyn-Y-Capel Site

There are very few early references for the site. The first known recorded reference is an Elizabethan survey of 1562 which described it as "Sancte Bride from Barfro (Aberffraw) iiij miles a creke for small pickards" (Baynes, 1921). The site is clearly indicated as a chapel on Saxton's map of 1578 (Figure 1.3), Speed's map of 1610 (Figure 1.4), where it is called "Cap Llanfanfraidd", and on Collin's map of 1693 (Figure 1.5), as "St Ffraid chaple". There is a print of the site, dated 1776 by Moses Griffiths in Pennants Tours in Wales (Pennant, 1778; Figure 1.6) which shows the ruins of a stone building standing almost to eaves height, with an east window, the remains of a south window lighting the sanctuary and a south door at the west end. Unfortunately, it is not possible to recognise any further architectural detail.

The Chapel is shown situated on the east end of a high isolated mound with a raised track passing between the mound and the tidal inlet on the east side. The sides of the mound are depicted as very steep slopes, particularly at the east end, and although it is possible this was accentuated in the drawing, Pennant, who in all likelihood visited the site with Griffiths, offers this description.

"Go over Towyn y Capel, a low sandy common, bounded on one side by rocks, which in high winds the sea breaks over in a most awful and stupendous manner, and are justly dreaded by mariners. In the middle of the common is an artificial mound, on which are the ruins of Capel St. Ffraid. I have no doubt that, prior to the chapel, it had been the site of a small fort, for I never saw the artificial elevations given to any but works of a military kind". (Pennant 1781).

The dimensions of the Chapel are recorded as "about thirty or thirty five feet by twenty two feet six inches". The walls were four feet thick, and the foundations extended to a depth of eleven feet into the mound (Stanley, 1846). The mound was 31 feet above the surrounding sward, and 36 feet above the shore. The top was 50 feet in diameter, and the diameter at the base was 250 feet.

Figure 1.3

Figure 1.4

Figure 1.5

Figures 1.6, 1.7, 1.8

The graves in the mound were arranged in four or five tiers, and were mostly cist graves, although plain burials were also found at the site. Approximately one third of the mound had been washed away by 1846, including the west end of the chapel (Stanley 1846) (Figure 1.7). In a later article, Stanley (1868) records the mound as having wholly perished.

A series of articles in *Archaeologia Cambrensis* and the *Transactions of the Anglesey Antiquarian Society* (Llywd, 1833; Baynes, 1921; Baynes, 1928; Thomas, 1937; Thomas, 1938) record the continued erosion of the mound, and the exposure of large numbers of burials. In 1980 a bronze pernacular brooch dated to the 8th or 9th century AD was found during the strengthening of the sea wall close to the mound (Lewis, 1982) (Figure 1.8).

1.1.1 Exploratory Excavation and Initial Observations

In 1997, excavations by Gwynedd Archaeological Trust (GAT) were initially carried out to assess the archaeological value of the burial mound. A trial excavation was performed using a trench of approximately 2 m wide by 9 m long into the seaward side of the Towyn-Y-Capel mound. The trench was adjacent to an upright slab that was interpreted as part of a cist grave.

The principal stratigraphic divisions were two prominent dark turf lines within the sand, both sloping up from the east to west, towards the former top of the mound, and separated by some 1.4 m of sand. Both turf lines were clearly truncated on the western side of the mound. In between the two turf lines, the sand was divided into fine and coarse layers, indicating times when storms would have been heavier, and thus would have transported and deposited the larger material. There were nineteen identifiable layers between the two ground surfaces, and seven below the lower turf line.

Two types of burial were found; stone lined cist graves, which had stone sides, ends and tops but no bases (Figure 1.9), and simple dug graves. There was no indication of any archaeology lower than cists, which lay at approximately 5.5 m OD. In the two cist graves six individual remains were found, 5 children and one adult, a femur was carbon dated to approximately 555 -885 AD (Figure 1.10) (Table 1.1).

The simple dug burials lay between the upper and lower turf lines in two layers, of which only one was found in the lower level amongst the cist burials. One hundred and three skeletons were found in dug burials, ranging from the remains of a child of 4-5 to the remains of an adult over 45 (Figures 1.11 & 1.12). A femur submitted for radiocarbon dating gave a date of between 1030 and 1220 AD for this burial (Table 1.1).

Figures 1.9 & 1.10

Figures 1.11 & 1.12

The skeletal remains of a number of other burials were found in the upper layers of the mound. These were all very disturbed and consisted of jumbled bones. They were interpreted therefore as reburials of bones that had formerly been eroded away from the mound.

1.1.2 Full Site Excavation of Towyn-Y-Capel

Due to continuing erosion, the complete excavation of the Towyn-Y-Capel site was planned and executed by the Gwynedd Archaeological Trust (GAT) (Excavation site ID GAT 1746) in conjunction of staff and students from the School of Forensic and Investigative Sciences, UCLan and the University of Cardiff. The principal investigator was part of this team and the work in this thesis began with this excavation. The burial mound was completely excavated over two periods, totalling 3 months work over 2 years. Soil contexts, finds, burials and skeletons were numbered sequentially and cross-referenced where possible.

Because of the unstable nature of the site soil (sand) and the likelihood of section collapse, sequential excavation of the whole site was undertaken through stratigraphic layers, commencing with the removal of modern windblown sand back to the dominant 18th Century AD turf line discovered during the initial excavation in 1997. This required the removal of some 200 to 300 tons of blown sand (approximately 1.5 m thick across the site), and revealed the turf layer (context 126) whereupon the ruins of the medieval Chapel still stood in18th Century AD (Figure 1.13)

Underneath the turf layer the remains of a stone wall encircling the mound, thought to represent part of the boundary of the cemetery described by Stanley in the 19th Century AD. The angled stones, leaning against and upon those of the cemetery wall, most likely collapsed masonry from the Chapel, particularly as some were associated with small patches of mortar (Figure 1.14).

Once the windblown sand had been removed, and excavations had progressed to the upper turf line (context 189) underlying the stone wall, the top of the mound was cleared to the first layer, and the turf line and underlying sand removed. This revealed the first series of burials, many of which were severely truncated or disturbed, and none were complete. There was also evidence of reburial of bones which had previously been eroded (Figure 1.15). Excavation proceeded to the next layer where complete inhumations were uncovered, all from simple dug graves, although the nature of the deposits made it impossible to recognise the grave cuts. Some inter-cutting of burials (Figure 1.16) had occurred, suggesting a reasonably long period of use.

Figure 1.13 & 1.14

Figure 1.15

Figures 1.16 & 1.17

It is thought that dug burials date to the 12th Century AD, which is somewhat supported by the radiocarbon dating (Table 1.1). These burials were all in simple dug graves. Of the one hundred and three dug grave burials recorded from the upper phase six were submitted for radiocarbon dating. Over all the burials were in three distinct burial phases according to the depth and, although apparently unmarked, the majority were carefully laid out as though to avoid earlier burials. Many of these were infant burials (Figure 1.17), and a number of the burials were incomplete because of erosion on both sides of the mound. The infant burials appeared to form a distinct cluster on the southern side of the site. The adult burials revealed several different methods of laying out the body, including a group of three individuals that were buried with their knees upright and their legs flexed.

In the lower phase of the burial context in between the upper and lower turf layer (between contexts 158 and 159) lay 24 stone lined cist graves were excavated, and found to contain both juvenile (11 individuals) and adult remains (10 individuals), 4 of which were sampled for radiocarbon dating (Table 1.1). Though variations existed, the cist graves typically consisted of a stone cist side and lintel but no basal slabs, buried in a rectangular pit approximately 1 m deep (Figures 1.18 & 1.19). The cists were generally carefully constructed of large tooled side slabs.

The bodies in the cists were usually fully extended inhumations, although the legs were, on occasion, slightly flexed. Following construction of the cist and burial, the grave above was backfilled, and a low mound created on the surface surrounded by a ring of boulders. The accumulation of wind blown sand over the burials shortly after their conservation preserved the above ground grave markings in excellent condition. The skeletons within the cists were well preserved, and two of the skeletons had hair remaining, one in long lengths over the shoulder and down the front of the body.

Underlying the cist burials was a distinct 0.3 m thick turf layer (context 189), of dark humic sand, that predates the burials. Small quantities of animal bone from cattle (*Bos)*, pig (*Suis*) and sheep (*Ovis*) were found with signs of butchery (straight edged cuts) which indicated human use. Charcoal recovered from this layer was dated to AD 540-660 (Table 1.1).

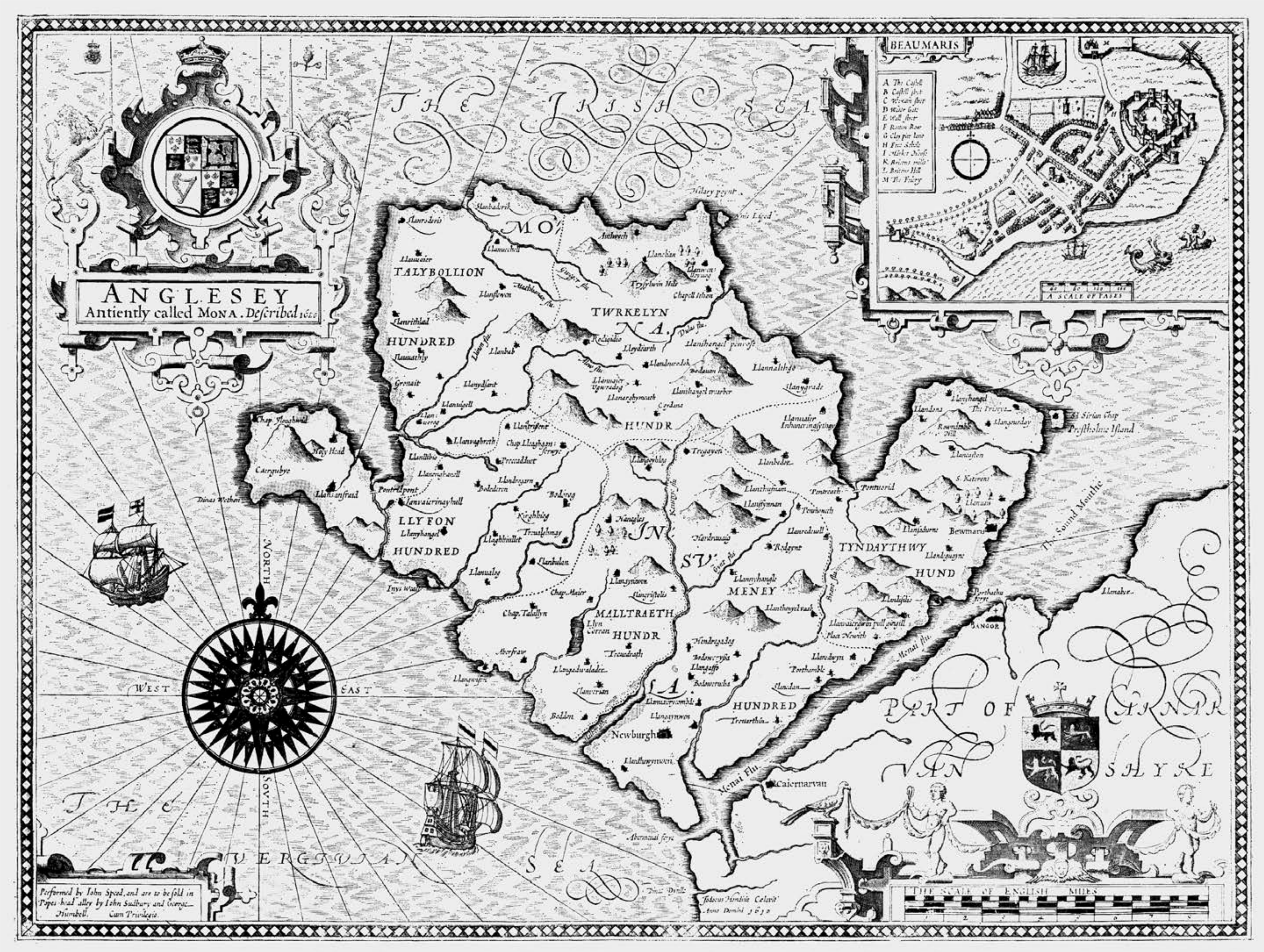
Further specific details on the archaeological excavation are available from the chief archaeologist Andrew Davidson of Gwynedd Archaeological Trust (GAT) although published material on the archaeological excavation itself and its association to other early mediaeval excavations on Holy Island are still forthcoming.

Table 1.1: Radiocarbon Dates from Towyn-Y-Capel in Chronological Order.				
Source Material	**Radiocarbon Age[1]**	**2 Sigma Range[2]**	**1 Sigma Range[3]**	**Source location or context**
B7	910 ±70	1000-1270 AD	1030-1220 AD	Dug Burial
Sk59	1120 ±70	770-1030 AD	870-1000 AD	Dug Burial
Sk34	1156 ±26	781-976 AD	783-959 AD	Dug Burial
Sk51	1180 ±50	710-980 AD	780-900 AD	Dug Burial
Sk60	1238 ±26	690-881 AD	694-859 AD	Dug Burial
Sk105	1262 ±26	676-858 AD	692-777 AD	Cist 214
Sk33	1270 ±60	650-890 AD	680-790 AD	Dug Burial
Sk102	1281 ±25	673-777 AD	690-770 AD	Cist 213
Sk108	1290 ±50	650-870 AD	670-780 AD	Cist 212
B10	1350 ±90	555-885 AD	635-775 AD	Cist 292
Soil	1450 ±40	540-660 AD	N/A	Context 189

Radiocarbon data indicates that the dug burials span between 650 and 1270 AD, although taking into consideration error margins and stratigraphy more likely to correspond to 700-1000 AD. In contrast, cist burials appear to be tightly grouped between 650-870 AD (if we ignore the anomalous cist 292/B10 and its large error margin). [1]Conventional radiocarbon age plus error in year before present (BP), [2]Calibrated to 95.4% probability using 2 sigma statistics according to Stuvier et al., 1998a/b, [3]Calibrated to 68% probability using 1 sigma statistics.

Figures 1.18 & 1.19

Figure 1.3: Re-Engraving of Saxton's Map of 1578.
Original engraving by Cristopher Saxton in the "Atlas of England and the counties of Wales, London 1579. British Library Special Collecton Hunterin Di.1.12. Re-engraved map by William Kip and William Hole from William Camden's 1637 edition of "Brittania", London 1607. British Library Special Collection Bn1-b1.


ANGLESEY
Antiently called MONA. Described 1610
THE IRISH SEA
BEAUMARIS
A SCALE OF PASES
TALYBOLLION
HUNDRED
TWRKELYN
HUNDR
LLYFON
HUNDRED
MALLTRAETH
HUNDR
TYNDAYTHWY
HUND
MENEY
HUNDRED
Holy Head
Newburgh
Bewmaris
BANGOR
Caernarvan
Menai Flu
NORTH
SOUTH
WEST
EAST
PART OF CARNARVAN SHYRE
THE VERGIVIAN SEA
THE SCALE OF ENGLISH MILES
Performed by John Speed, and are to be sold in Popes head alley by John Sudbury and George Humbell. Cum Privilegio.

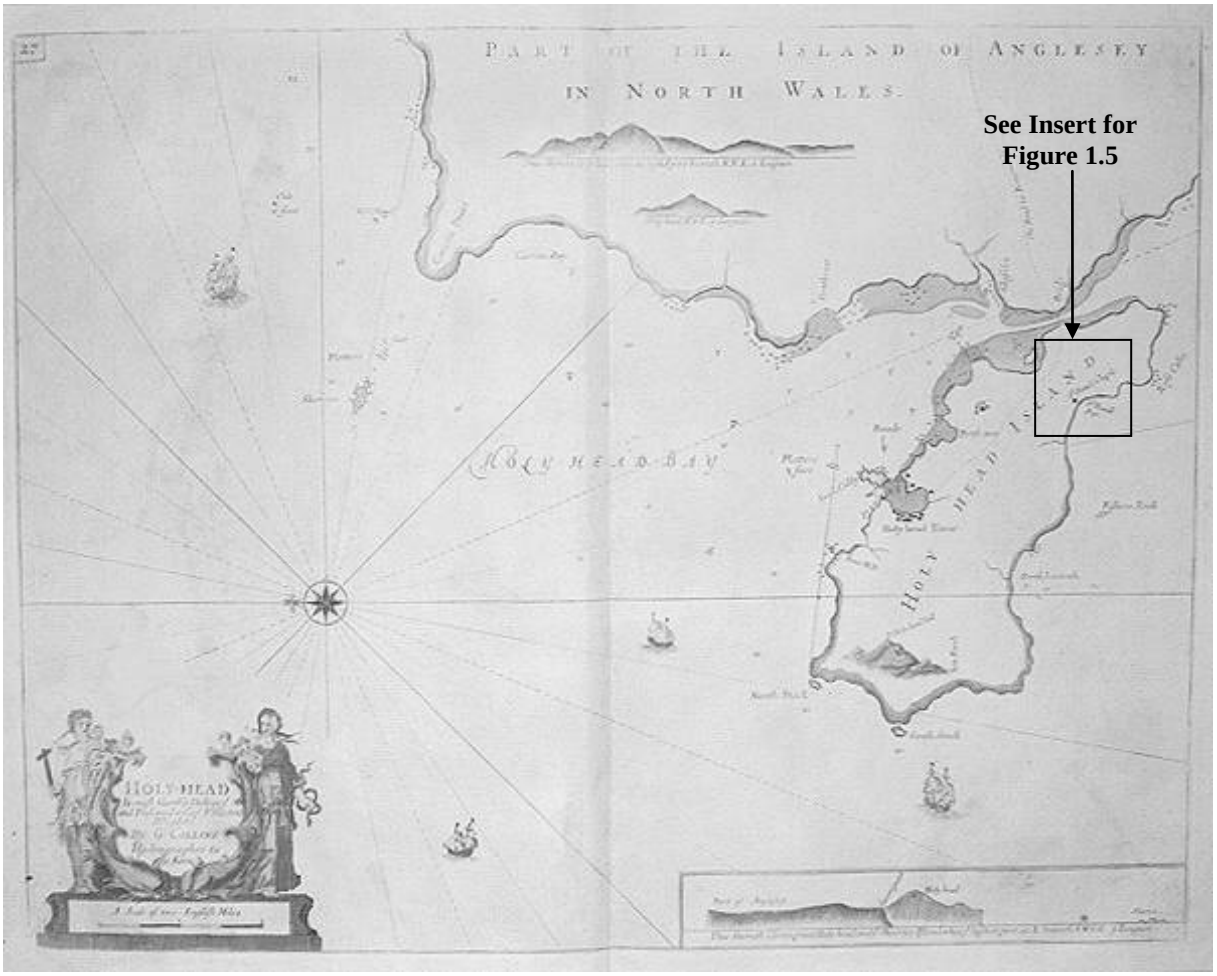


Figure 1.4: Speed's Map of 1610.
(Previous page)
From John Speed's atlas "The theatre of the Great Britaine: presenting an exact geography of the kingdoms of England, Scotland, Ireland and the isles adjoining." London, 1611. British Library Special Collection e140.

Figure 1.5 a: Collin's Map of 1693.
(Above) From Capt. Greenville Collins' "The Great Britain Coasting Pilot". London, 1693. The most detailed early illustration of the burial mound.

Figure 1.5 b: Insert of Collin's Map of 1693.
(Left) Shows an expanded view of the area marked in Figure 1.5a with detail of "St. Ffraid's Chaple" in "St.Ffraid's Bay" (Trearddur Bay).

Figure 1.6: Moses Griffith's Print of Capel St. Ffraid (Above). Early Print of the still intact Capel on the elevated burial mound from the 1776 print in Pennant's Tours in Wales (Pennant, 1781).

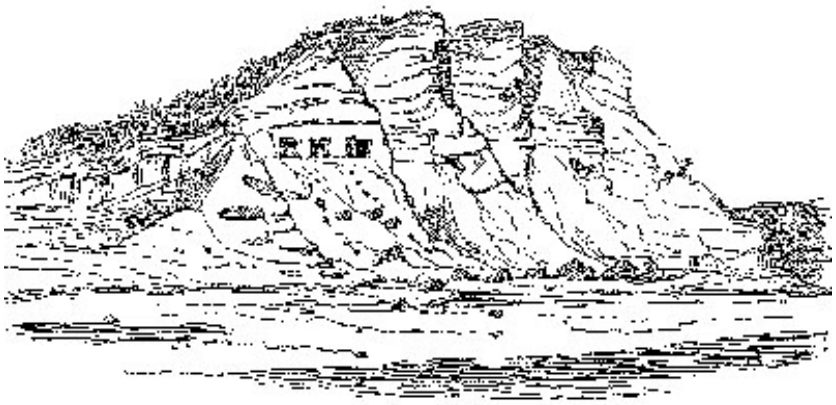


Figure 1.7: Sketch of the Towyn-Y-Capel Burial Mound(Centre).
Burial mound showing exposed cist graves, mid to late 19th Century. (Stanley 1868).

Figure 1.8: Photograph of Towyn-Y-Capel Burial Mound (Below).
South East facing photograph of Towyn-Y-Capel burial mound showing St Ffraid cross, sea wall/ boardwalk and Trearddur bay before the 2002 excavation.

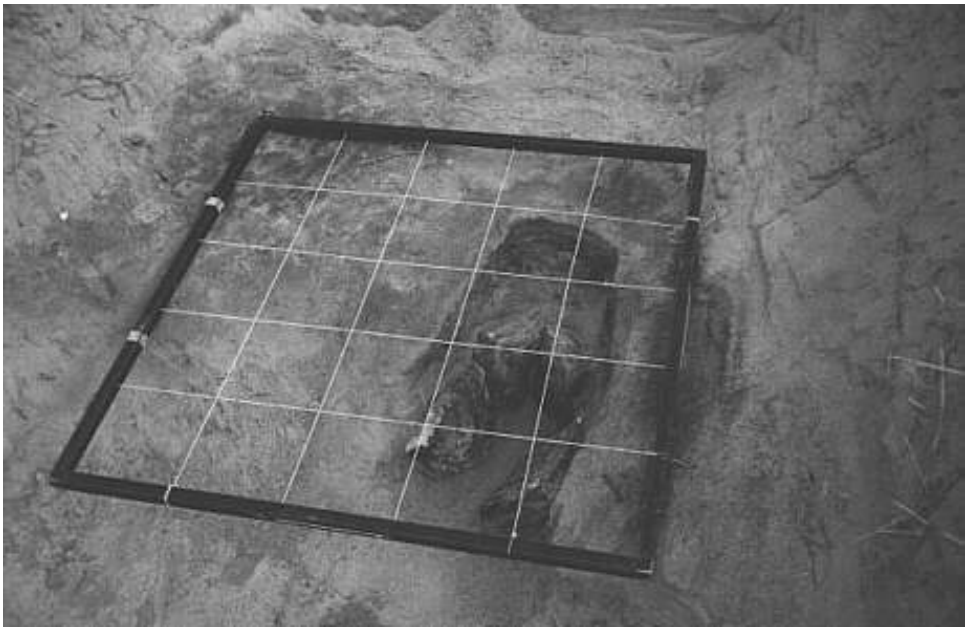


Figure 1.9: Photograph of Cist 1, 1997 (Above).
A small child cist (stone lined) grave uncovered during the trial excavation of 1997. Photograph courtesy of GAT.

Figure 1.10; Photograph of Cist 2, 1997 (Below).
Large adult cist grave, although intact the skeletons found within were severely disturbed and may not be associated with the original burial. Photograph courtesy of GAT.

Figure 1.11: Photograph of 45 year old Male Skeleton 1, 1997.
Partial skeleton found just below the 18th century turf Layer (context 158; 1 m down). Only the upper half of the skeleton is *in-situ*. This is representative of many of the upper level burial between the first and second turf layers and shows that erosion of the sand occurred at the turn of the century as supported by written documents. This erosion led to undermining of the burial mound leading to partial collapse of parts of the burial mound and skeletal disturbance.

Figure 1.12 Photograph of 45 year old Male Skeleton 2, 1997.
Complete excavation of the area surrounding the skeleton, shows that he was probable buried a shallow grave, not long before the chapel collapsed, as can be seen by the collapsed stone in the background of the photo.

Figure 1.13: Photograph upper (18th Century) turf layer, 2002.
The upward sloping (north to south) turf layer indicates that this is the north edge of the original burial mound. Most of the burials were found at the apex of the remaining mound as seen above.

Figure 1.14: Photograph of disturbed skull upper burial, 2002.
One of the many disturbed skeletons recovered from the upper burial levels. Skull found severely crushed under rock. The skeletal damage was probably caused post-mortem by a combination of erosion and the collapse of the chapel stones on to the soft sand burial.

Figure 1.15: Photograph of Collapsed Stones from St. Ffraid Chapel, 2002.
A large quantity of masonry stones recovered from across the apex of the modern day mound. This indicates the possible edge of the original burial mound. The mortar associated with some of the stones and the cut of the stones themselves indicate stone dressing for a building, probably the chapel itself.

Figure 1.16: Photograph of Inter-cutting Dug Graves, 2002.
Skeletons excavated during phase 1 in simple dug burials in between the first and second turf layer.

Figure 1.17: Photograph of Juvenile Skeleton in Dug Grave, 2002.
A large percentage of the burials above or around the first turf layer were juvenile skeletons.

Figure 1.18: Photograph of Cist 3, 2003.
North-South facing cist grave prior to skeletal excavation. This cist grave was compromised by the collapse of one of the stones and the burial chamber flooded with sand.

Figure 1.19: Photograph of Cist 4, 2003.
A deep cist grave showing a particularly good skeleton with excellent preservation. This skeleton was associated with a lot of deep root plant material. Hair samples were also recovered from this skeleton.

THE STRUCTURE OF FOSSILISED REMAINS

1.2 Hard Tissues; Structure and Organization

The branch of physical anthropology which deals with the scientific study of bones and teeth is known as osteology (White & Folkens, 2005). Osteology encompasses the structure of bones and teeth, their formation, morphology, function, pathology and ossification. The field is of particularly relevant to forensic, anthropological and archaeological investigations, as it can provide information such as biological age, gender, stature, pathology and, in some cases, further identifying information such as geographical origin and cause of death (ibid). Analysis of the Towyn-Y-Capel skeletal remains included a comprehensive examination and recording of the skeletal assemblages, which will be alluded to do at various stages within this thesis, particularly with regard to their preservation and in comparison with the results of molecular experiments and the individual skeletal elements.

1.2.1 Gross Anatomy of Osseous Tissue

The bones comprising the human skeleton, at first glance appears to be made up of a wide range of shapes that appear to be extraordinarily diverse. However, the bones of the body may be divided into a few basic but overlapping shapes; the bones of the limbs (including those of the hands and feet), also called long bones, are tubular with expanded ends; the bones of the cranial vault, shoulder, pelvis and rib cage tend to be flat and trabecular; and the bones of the ankle, wrist and spine are blocky and irregular. In addition to these three basic external shapes, bones may also be classified on the basis of their internal structures into either cortical (compact) or cancellous (spongy) bones (Figure 1.20) (White & Folkens, 2005).

Cortical bones make up the walls of bone shafts and external bone surfaces whereas cancellous bone is found in protuberances where tendons attach, in vertebral bodies, at the ends of long bones, in short bones and sandwiched between flat bones (ibid). Cancellous or trabecullar bone has a spongy, porous and lightweight honeycomb structure, and named after the thin bony spinicules (called trabeculae) that form it. Despite the structural differences in porosity, the cellular and molecular compositions of the bone tissues are identical. The porosity of the trabecullar bone provides a reservoir for the red marrow, a blood forming or homeopoietic tissue that produces red and white blood cells and platelets. Yellow marrow found in the medullary cavity of tubular bones functions mainly as a reserve of fat cells, and gradually replaces the red marrow in most long bones (White & Folkens, 2005).

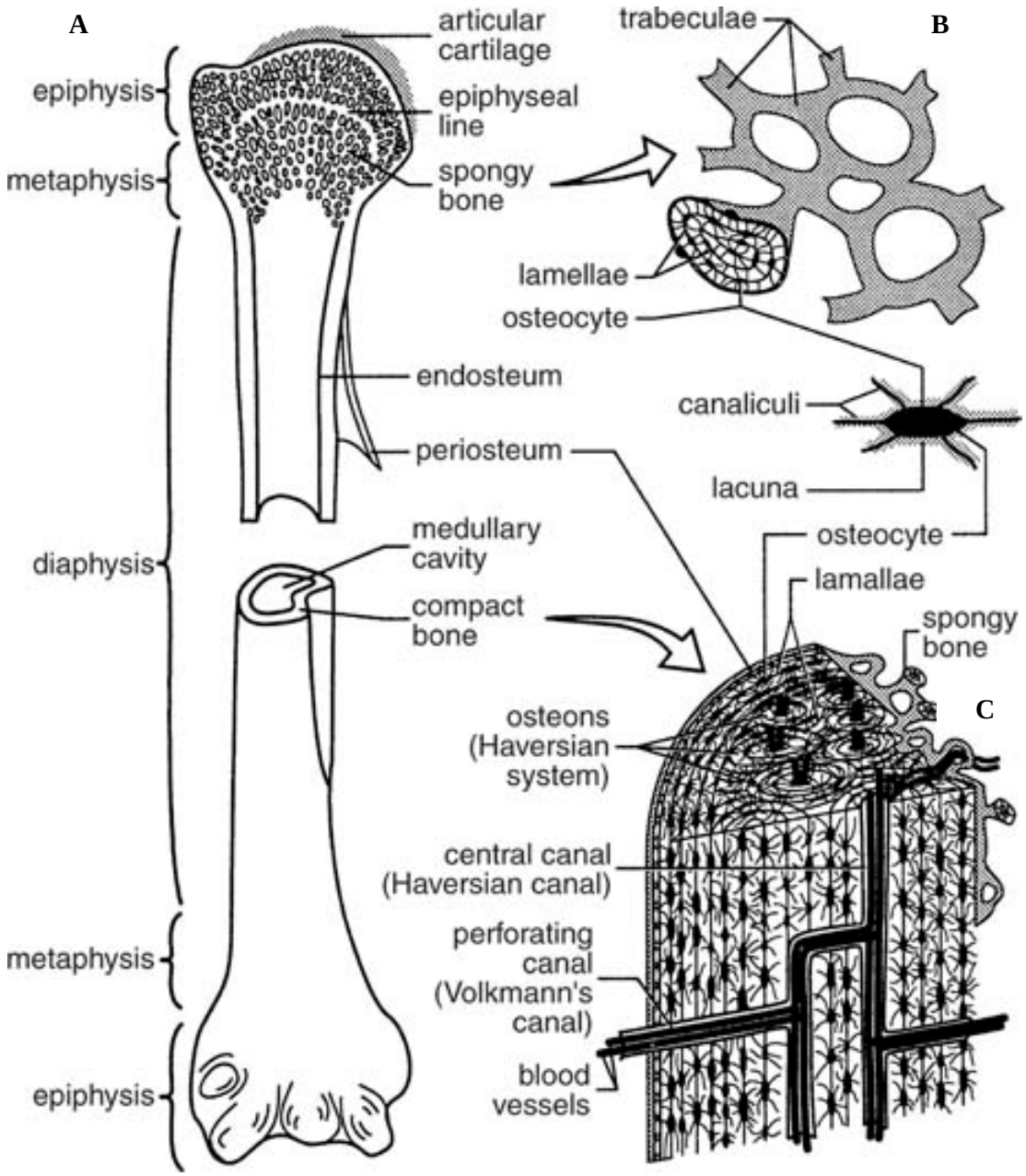


Figure 1.20: Gross Anatomy, Structure and Composition of Bone.
Shows the three different parts of bones; diaphysis, metaphases and epiphysis (A), the two types of bone tissue, spongy and compact bone and the vascularised membranes lining the inner (endosteum) and outer (periosteum) part of the bone.(C) At the Microscopic level it shows the form of the lamellae and component osteocytes in both spongy (trabeculae) (B) and compact bone, with further detail of the osteons (Haversian System) and the associated systems and canals (central and perforating or Volkmann's) in the cortical (compact) bone (C). Figure taken from www.cliffsnotes.com.

The Long bones can be divided into two regions according to form, function and origin, identified as the epiphysis and diaphysis. The epiphysis arises from secondary ossification of bone and forms the trabecullar structure at the articular surfaces of the bone. The diaphysis results from the primary ossification of the bone and forms the cortical bone structure. The two are bound by the metaphysis, represented by the expanded or flared end of the cortical bone (Figure 1.20). In living bone tissue the outer surface of the bones are covered with a thin layer of tissue called the periosteum, a vascularized membrane whose thin fibres nourish the bone by penetrating the outer surface and that also helps to bind the muscles to the bone. In addition to the outer membrane, a similar cellular membrane called the endosteum covers the inner surface of the bones (White & Folkens, 2005).

1.2.2 Histological Structure and Composition of Osseous Tissue

The study of tissues at the microscopic level is known as histology (Ross & Pawlina, 2010). As previously stated, bone tissues, despite variation in structural and morphological characteristics are essentially made up of the same histological type. In mammalian bone the main histological differentiation is between mature and immature bone types. Immature bone, also known as coarse bundles or woven bone, is the preliminary bone form, a phylogenetically primitive bone type of non-orientated collagen fibres that characterises the embryonic skeleton (Cormack, 2001). The mature bone type, or lamellar bone tissue, is a highly organised structure that is produced by the continuous addition of uniform lamellae onto the bone surface during appositional growth (Figure 1.20).

Collagen fibre orientation in bone alternates between layers giving the bone a plate-like structure or lamellar appearance. In compact bone there are three common patterns; cylindrical arrangements around vascular channels of varying size and number known as osteons; irregular areas between osteons called interstitial lamellae and lamella arrangement at the surfaces of the bone, following much of the circumference of the diaphysis (circumferential Lamellae) (White & Folkens, 2005).

Both compact and trabecular bones are made of lamellar bone tissue, although in compact bone the cells cannot be nourished by diffusion from the surface blood vessels due to the bone density. Haversian systems, with their canals and canaliculi, resolve these issues in compact bone. The different components and their cellular constituents are considered independently as they are imperative for the understanding of bone histology (Cormack, 2001).

The average osteon diameter is around 300 μm and is generally 3-5 mm long (Mathews, 1980) and is relatively uniform. Mineral density is variable due to the modelling and remodelling processes. Osteons appear as concentric arrangements around a circular opening when viewed in transverse section and as parallel layers that follow the path of the central vascular space when seen in longitudinal section. This arrangement of lamellae is common in compact bone and is called the Haversian system. Concentric lamellae are noticeably less visible in younger individuals, the classic appearance of the Haversian system visible mainly in the stable remodelled osteon of the adult skeletal tissue (White & Folkens, 2005).

The osteons formed during the initial growth of the bone are called primary osteons and those formed during remodelling, secondary osteons (Figure 1.21). Interstitial lamellae are irregularly arranged, with the majority of the bone appearing as a complex mosaic of rounded lamellar structures interconnected with more angular areas of lamellar matrix. Cement lines bound both osteons and interstitial lamellae and appear as thin refractile layers under microscopical investigation (Figure 1.22) (An et al., 2003).

Approximately 22-110 μm in diameter (Fawcett, 1994), the Haversian systems are clearly distinguishable from other blood vessels by their intimate association with the concentric arrangement of the osteon lamellae in which they are found. Haversian canals contain the blood vessels, capillaries and postcapillary venules. Volkmann's canals differ from the Haversian canals in not being intimately associated with the osteons, but instead penetrate the bone perpendicular to the pattern of lamination and provides connections between the blood vessels in the marrow or periosteum and those in the osteons. In order to accomplish this, the Volkmann's canals are larger than the corresponding Haversian canals (Figure 1.23).

Osteocyte lacunae are the cavities that hold the bone cells within the bone matrix. Roughly lenticular in morphology they are spaced almost evenly throughout the matrix (estimates place 25 000 of these elements within each cubic mm of bone) (Baron, 1993). The morphology of the osteocyte lacunae generally reflects the shape of the osteocyte contained within and although they are lined with a matrix of unmineralised collagen, they are enclosed by a heavily mineralised layer of bone matrix that is clearly visible under high power microscopy (Figure 1.24). The panosteocytic space, filled with extracellular fluid, lies between the osteocytes plasma membrane and the matrix wall in both the lacunae and canaliculi, where it plays a role in chemical exchange. The branching tunnels of the canaliculi provide passage for the numerous cell processes between neighbouring osteocytes within the lacunae. Existing as both long and short processes, they are often difficult to observe under light microscopy, although the longer processes can easily be seen under electron microscopy.

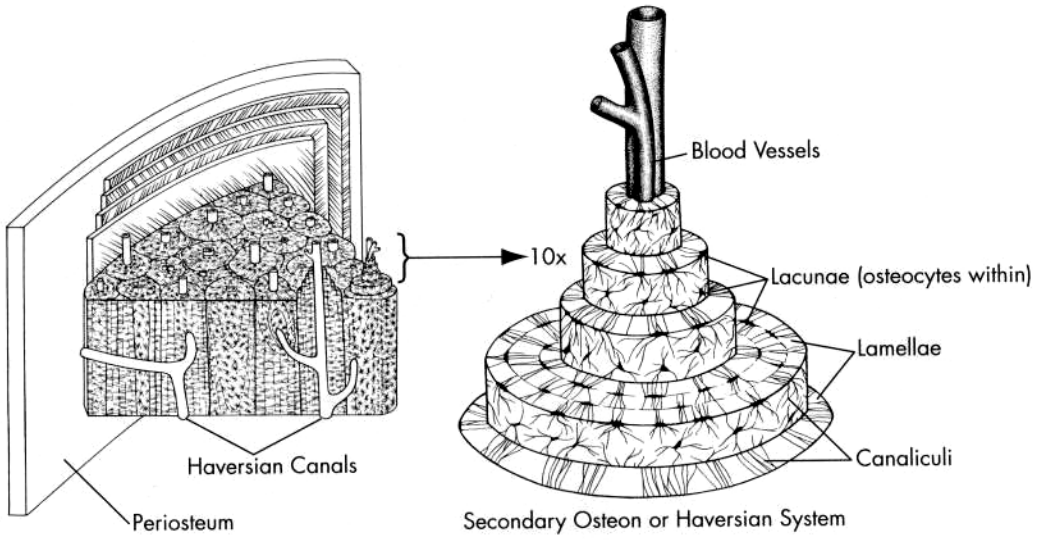


Figure: 1.21: Specific Microscopic Structure of Cortical Bone.
Shows the layout of cortical tissue with specific reference to the individual mature osteon unit (Secondary osteon or Haversian system). Image from White & Folkens, 2005.

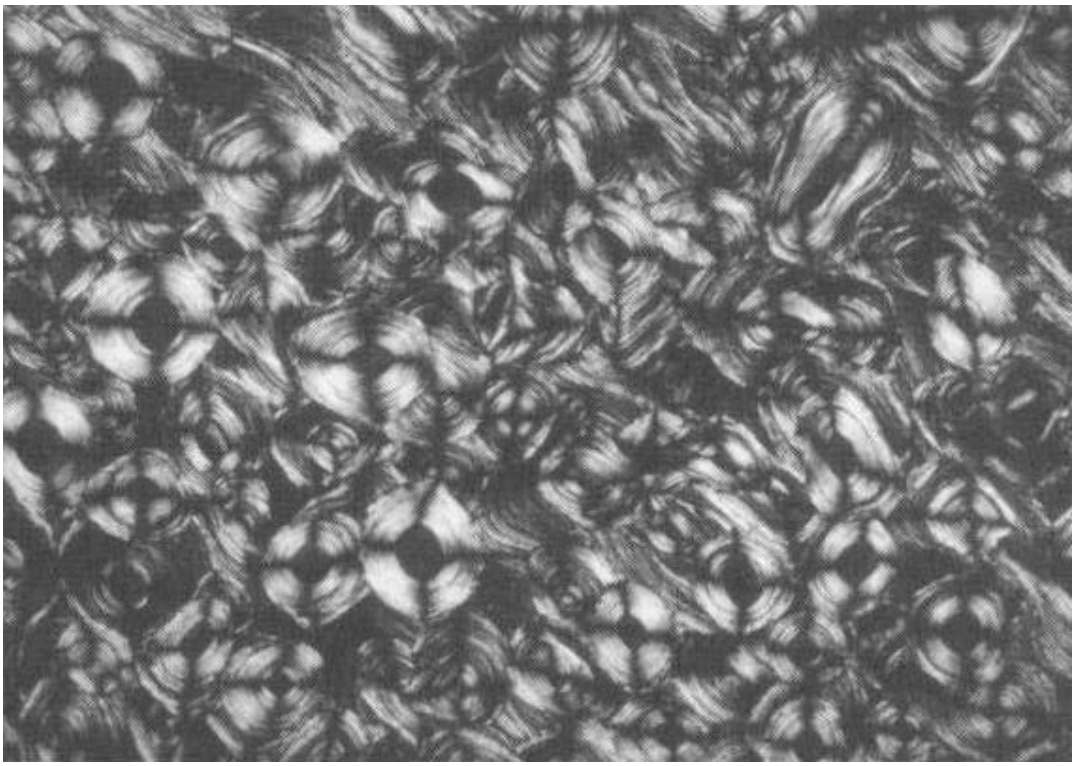


Figure 1.22: Microscopic Image of Osteons in Mature Human Bone.
Figure shows a particular dense and mature cortical concentration of Haversian systems, and a Volkmann's Canal in the upper right hand corner of the image. Image from Schultz, 1997.

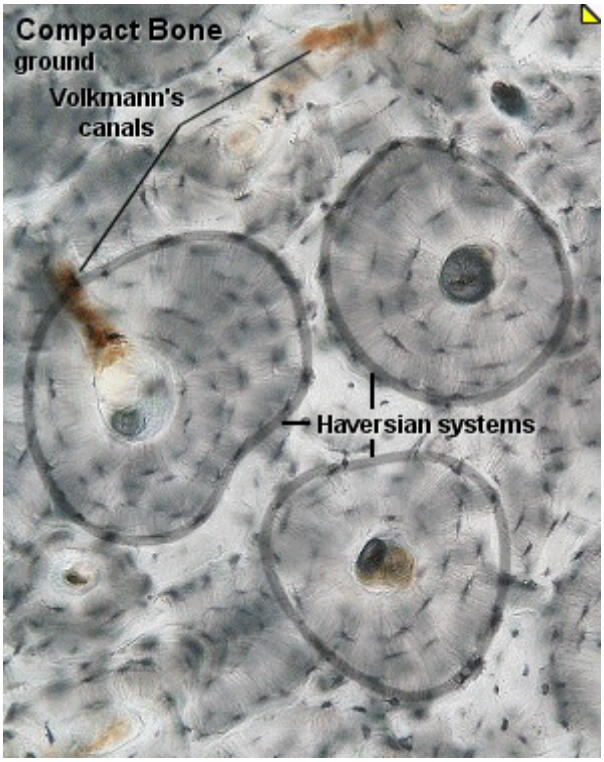


Figure 1.23: High Power Microscopic Image of Haversian Systems and Volkmann's Canals. Magnification x200. Image from www.lab.anhb.uwa.edu.au.

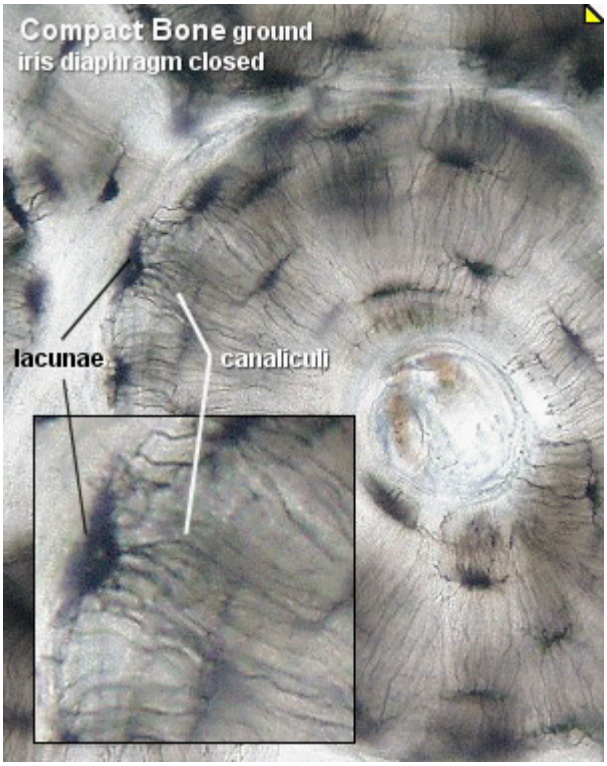


Figure 1.24: High Power Microscopic Image of Osteon detail, including Lacunae & Canaliculi. Magnification x400 (insert magnification x1000). Image from www.lab.anhb.uwa.edu.au.

The periosteum is varied in appearance under microscopy, due to environmental and functional factors. Bones develop due to an inner layer of osteoblasts in contact with the bone surface. Here, osteoblasts revert to lining cells, a type of connective tissue, once growth has been arrested. They can revert to their original function in order to repair or consolidate the outer bone structure. The outer part of the periosteum is relatively free of cells and is largely formed of connective tissue supporting networks of blood vessels that function via the Volkmann's canals. During bone development these blood vessels and supporting collagen fibres can be incorporated into the outer layer of the bone matrix, called Sharpey's fibres, and act as anchors for the periosteum. Lining the inner surfaces of the bone is a thin membrane of squamous cells known as the endosteum, which has a similar bone repair function. These line all inner cavities in bones, such as trabeculae in the medullary cavity and the interior of blood vessels and canals. Both these tissues are osteogenic tissues that contain numerous bone forming cells which are active throughout the lifespan of the individual, and which play an active role in bone deposition and repair after severe bone trauma (Fawcett, 1994).

1.2.3 Osseous Cytology; Distribution & Function

Bone is the predominant biological tissue to be recovered from ancient remains. Its cells are intimately associated with the inorganic structure of the bone and hence are more aptly suited to withstand the ravages of time. The structure and distribution of these cells is instrumental in biomolecular investigations of calcified tissues. There are three major types of cells associated with bone, osteoblasts, osteoclasts and osteocytes (White & Folkens, 2005). A fourth cell type is found in the early stages of intramembraneous bone formation at the centres of ossification and therefore relatively undifferentiated. Osteoblasts are mononucleated cells responsible for bone formation, predominately through the secretion of an organic matrix, called the osteoid, pre-bone tissue that mineralises approximately 10 days later (Baron, 1993). As mononucleated cells found in the unmineralised matrix of the bone osteoblasts are of limited interest in biomolecular archaeology as they are less likely to survive in skeletal remains.

Osteocytes, alternatively, differ in their location, contained within the mineralised matrix of the bone and the osteocyte lacunae itself. The lacunae associated with newly formed osteocytes are initially lined with uncalcified collagen that later becomes more mineralised (Robinson et al., 1973). As the cell becomes further embedded in the mineralised matrix of the lacunae (Enlow, 1990), the cell itself begins to enter a stationary growth phase, and a series of ultrastructural changes takes place (Baron, 1993). Initially the endoplasmic reticulum

is reduced in size and function, mitochondrion and ribosome count is reduced, and there is an accumulation of lipid-like bodies and glycogen.

Finally, there is 'chromatin clumping' and uniformity of density across the nucleus (Robinson et al., 1973). In addition there is reduced lysosme activity in the aged osteocyte. Lysosmes are membrane bound organelles in which the digestive enzymes are located. The life span of an osteocyte varies but is generally considered to be about 7 years (Enlow, 1990).

Osteoclasts are multinucleic cells histologically identifiable by the degree of peripheral clumping of nuclear contents, ruffled border appearance of the outer membranes and high numbers of organelles. The cells are responsible for bone resorption and have specialised regions in their membranes and cytoplasm that are actively associated with this process. The cells are rich potential sources of mtDNA due to the numerous mitochondria. Unfortunately, the osteoclast lysozymes are also particularly rich in hydrolytic enzymes (Robinson et al., 1973) which include the DNAses and contribute to DNA breakdown during autolysis. Thus, although these are rich in mitochondrial DNA, these enzymes present difficulties in extracting nucleic acids for genetic analysis. Taken together with their structural location in bone and the relatively low numbers of these cells, osteoclasts are considered to be of limited potential for DNA extraction

1.2.4 Gross Anatomy of Odontological Tissues

Teeth constitute the part of the living skeleton that interacts directly with the environment, serving to seize and masticate food material for subsequent digestion and uptake (Hillson, 2002). The internal composition and external morphology of teeth are adapted to this function, which leads to the unique strength and preservation of dental material in archaeological terms. From the perspective of a Physical Anthropologist (Osteologist), the dentition is one of the most important parts of the human anatomy to be recovered. Their resistance to physical and chemical destruction means that they are over represented in almost all archaeological and paleontological assemblages. In addition teeth can provide particular information about the individual, including age, sex, health and diet (ibid).

The structure of human teeth varies depending on the individual tooth position and function within the jaw (ibid). The identification of the tooth position is not just important from an anthropological perspective but also from a biomolecular perspective, as different teeth emerge at different times, the correct identification of teeth is imperative to understanding the time frame of particular biological process, such as diet. In order to better understand the

research into teeth, their identification, selection and research observations. A brief overview of dental nomenclature, structure, cellular and molecular composition of dental tissues a brief overview of physical and biological properties of teeth is presented below.

In adult humans, teeth are differentiated by function into four types- incisors, canines, premolars and molars (Table 1.2, Figure 1.25) (ibid). Incisors consist of eight spatulate teeth in the front upper and lower jaw (2 in each Quadrant Figure 1.26). There are four canines (Figure 1.26) which are as posterior extensions of the incisor rows, but with a more conical and elevated shape. Premolars (or bicuspids), follow behind, four in each jaw (two pairs) (Figure 1.26). Depending on the nomenclature system being used, these may be classified as third and fourth premolars as first and second premolars have been lost in early hominid evolution. For simplicity, these were classified as first and second premolars in this study. The remaining teeth are made up of molars, the largest of the teeth with extensive chewing surfaces designed for crushing and grinding. There are usually six molars per jaw (in sets of three) (Figure 1.25-1.28) (ibid).

Designations used for this research are based upon the standard human osteological shorthand which is both unambiguous and straightforward. This denoting the sagittal plane ({L}eft or {R}ight), tooth type ({I}ncisor, {C}anine, {P}remolar or {M}olar), mesial to distal position (1-3 depending on tooth type, from the anterior of the jaw to the posterior) and whether they are mandibular or maxillary teeth (upper or lower jaw position) denoted by superscripting or subscripting, accordingly. Their specific positional numbers as shown in some in figure 1.27 (ibid).

Depending on the field of study and regional preferences however, alternate labelling systems are used in the literature, such as the quadrant based Zsigmondy System and the two digit code of the Federation Dentaire Internationale [1](FDI) System (ibid).

Teeth form in humans at a normalised rate that can be used to by osteologists to age individual. A brief overview of the human tooth formation/eruption timetable for permanent dentition is given in Table 1.3 as related to this research; primary dentition schedule and dentition are not being considered as it is outside the scope of this work.

[1] http://www.fdiworldental.org/

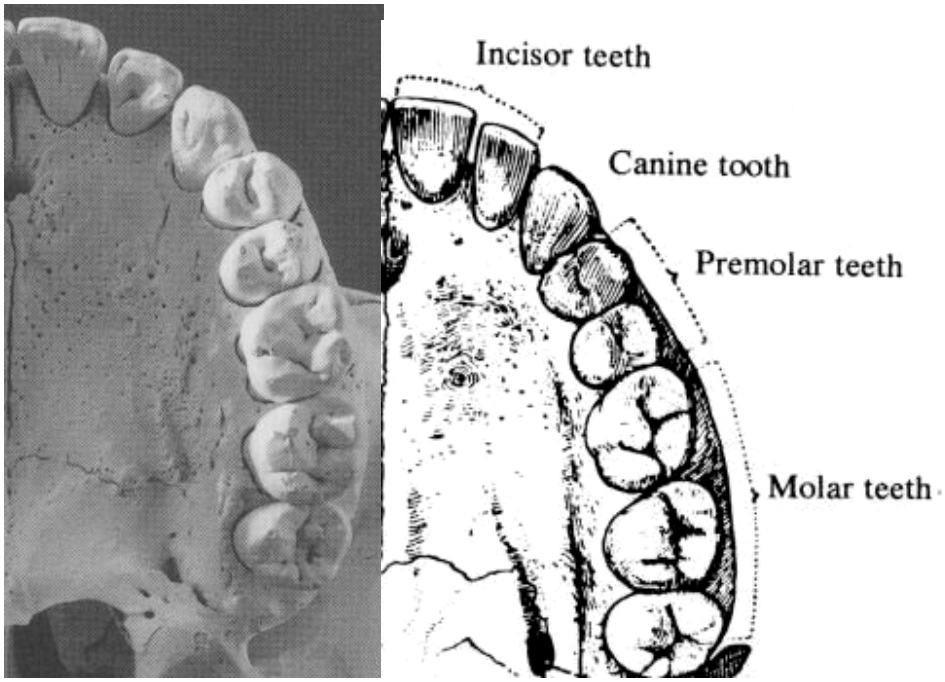


Figure 1.25: The 4 types of Human Teeth in the Left Upper Maxillary (a single Quadrant of the Upper Jaw). Composite image courtesy of Jennie Robinson.

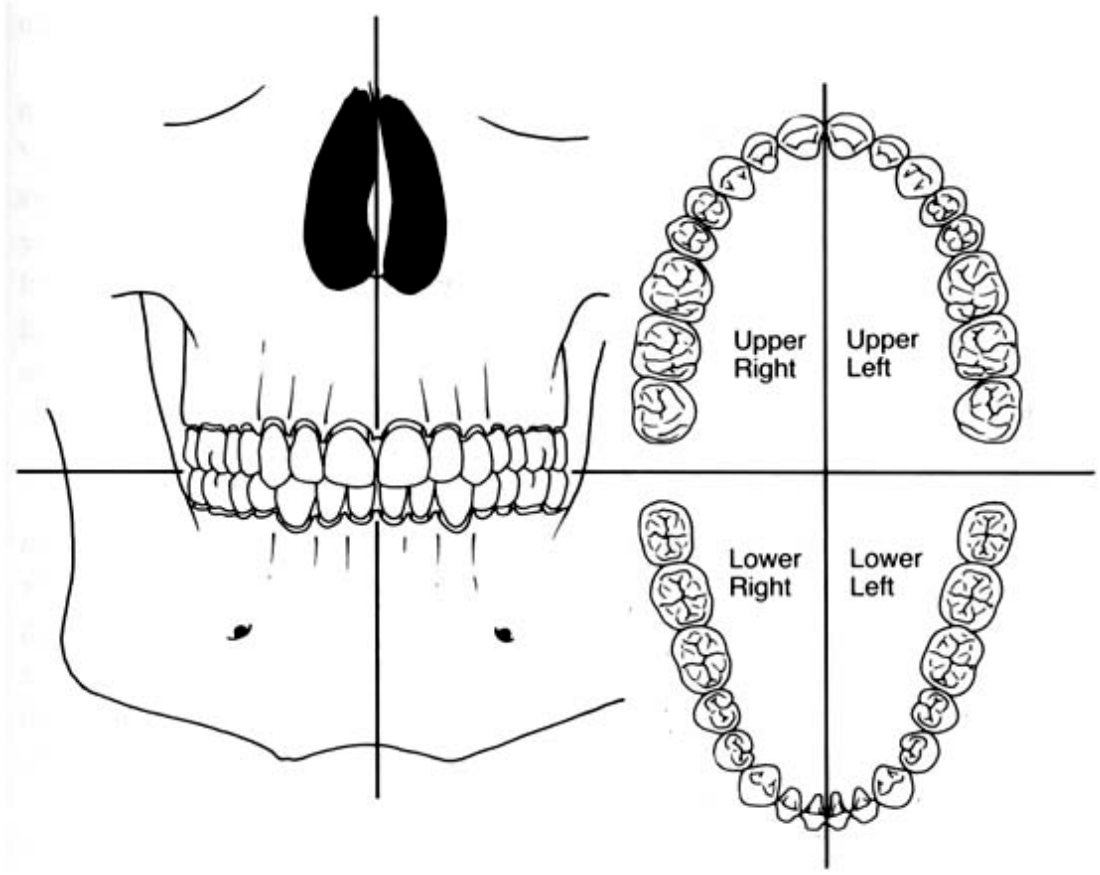


Figure 1.26: Quadrant Spread of Human Permanent Dentition across the Maxillary & Mandible (Upper and Lower Jaws). Image from Hillson, 2002.

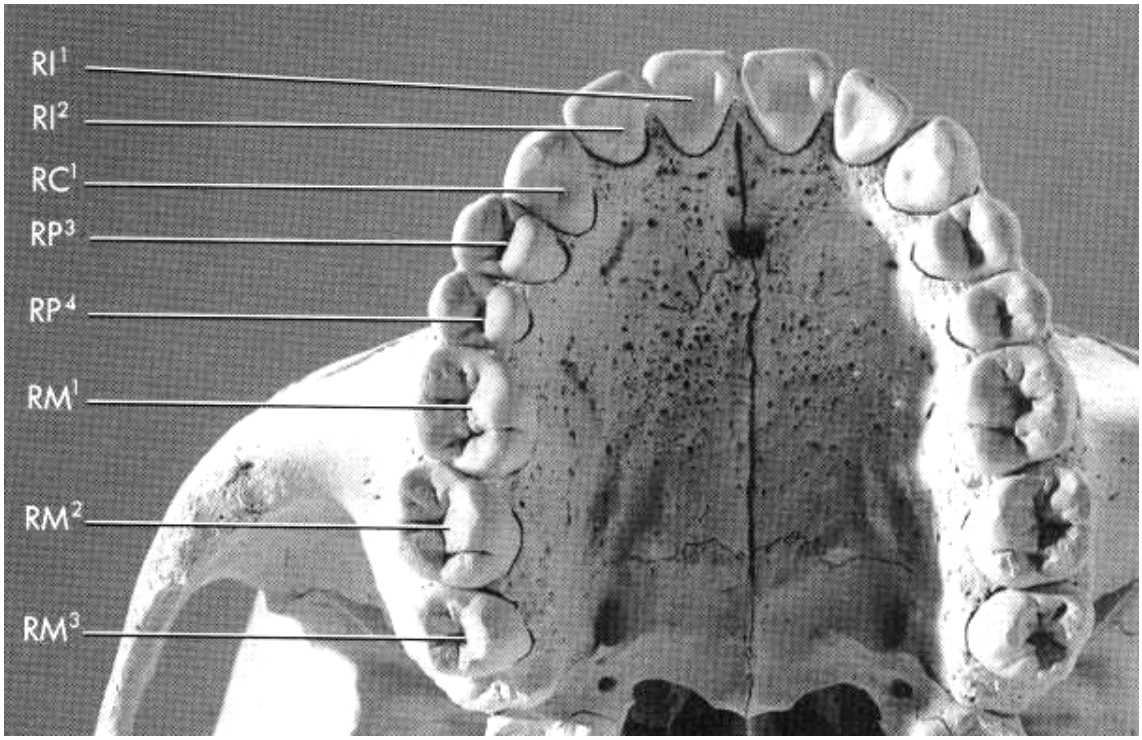


Figure 1.27: Layout of Permanent Dentition on the Right Maxillary (Right Upper Jaw).
Figure from White & Folkens, 2005.

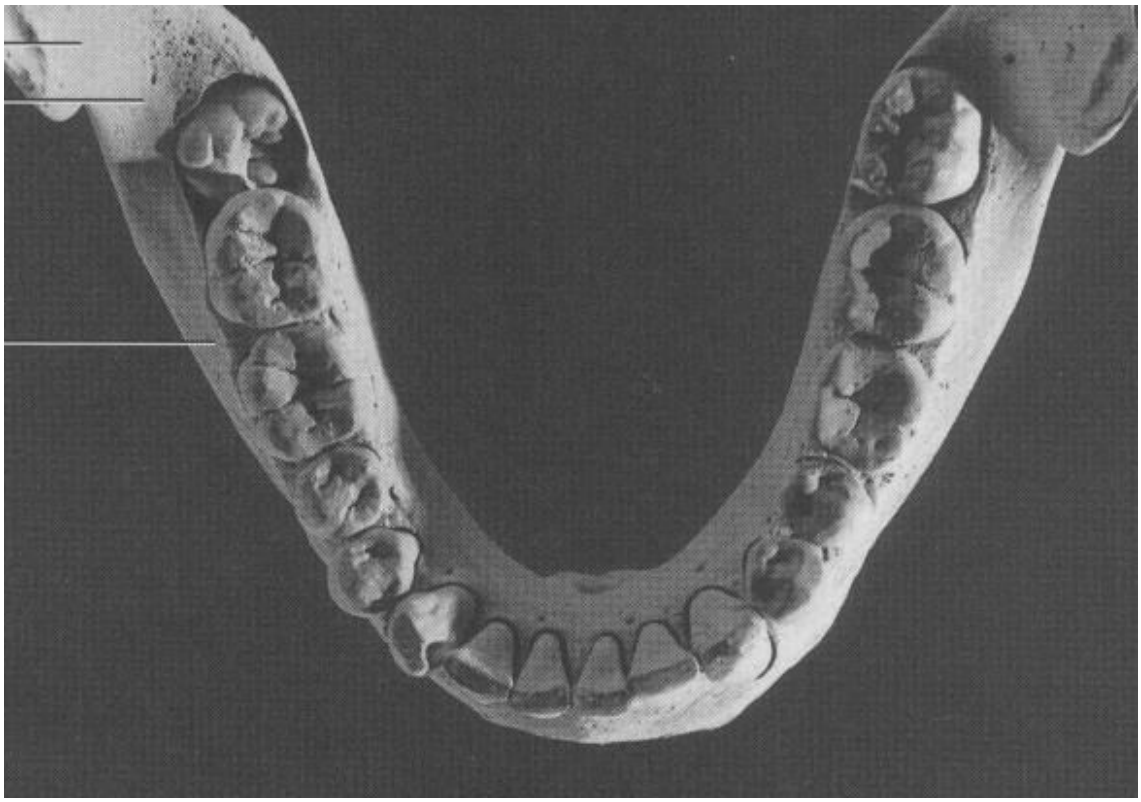


Figure 1.28: Layout of Permanent Dentition on the Mandible (Lower Jaw).
Figure from White & Folkens, 2005.

Table 1.2: Anthropological Designations and Description of Permanent Human Dentition				
Tooth Type	**Designation**			**Descriptions**
	Jaw	**Right Side**	**Left Side**	
Incisors (I)	Upper (Maxilla)	RI^1 RI^2	LI^1 LI^2	Blade-like front teeth that cut and shear food at the front of the mouth. Upper Incisors are limited to the premaxilla bone. Incisors are relatively small, simple teeth in most primates.
	Lower (Mandible)	RI_1 RI_2	LI_1 LI_2	
Canines (C)	Upper (Maxilla)	RC^1	LC^1	Large teeth at corners of mouth, distal to the incisors, which can pierce food and whose relative size important to social structure of many groups of primates. Upper canine is first tooth immediately behind suture between premaxilla and maxilla. Lower canine is tooth immediately in front of upper canine when upper and lower jaws occluded.
	Lower (Mandible)	RC_1	LC_1	
Premolars (P) or (PM)	Upper (Maxilla)	RP^1 RP^2	LP^1 LP^2	Intermediate in form between canines and molars; commonly have two cusps (raised points on the crown) so referred to as bicuspids. Often have a thickened ring of enamel around base of crown called the cingulum.
	Lower (Mandible)	RP_1 RP_2	LP_1 LP_2	
Molars (M)	Upper (Maxilla)	RM^1 RM^2 RM^3	LM^1 LM^2 LM^3	Expanded occlusal surface, with more cusps than premolars for crushing and grinding food. Upper molars of primates all derive from tritubercular (triangular-cusp) pattern. Crown of each triturbercular tooth has three main cusps 1) Protocone, 2) Metacone and 3) Paracone.
	Lower (Mandible)	RM_1 RM_2 RM_3	LM_1 LM_2 LM_3	

Designations indicated in this table are used throughout this thesis, Table adapted from information from White & Folkens, 2005 & Hillson, 2002.

Table 1.3 Important Features of Tooth Anatomy	
Crown	Part of the tooth covered by enamel.
Root	Anchors the tooth in the alveolus of the mandible or maxilla.
Neck (Cervix)	Constricted part of tooth at the junction of crown and root.
Enamel	Specialised hard tissue that covers the crown.
Cervicoenamel Junction CEJ)	Line encircling the crown, the most rootward extant of the enamel.
Dentinoenamel Junction (DEJ)	Boundary between the enamel cap and the underlying dentin.
Dentine	Core tissue of tooth, underlies enamel and encapsulates the pulp which supports the odontoblasts that line the pulp chamber.
Pulp Chamber	Expanded part of the pulp cavity at the crown end of the tooth.
Root Canal	Narrow end of the pulp cavity at the root end.
Cementum	Bone like tissue that covers the external surfaces of the tooth roots.
Calculus	Calcified deposit of plaque.
Pulp	Soft tissue of nerves and blood vessels within the pulp chamber & root canal.
Apical Forment	The opening of the root tip, or apex, where nerve fibres and blood vessels pass from the alveolar region to the pulp cavity.
Cusp	Occlusal projection of the crown. Major cusps in hominoid crowns are named individually. Essential in identifying loose lying teeth. Maxillae cusps have -cone suffix, mandibular cusps –conid suffix.
Protocone	Mesiolingual cusp, upper molar. Unique topographical features are called Carabelli's effects.
Hypocone	Distolingual cusp, upper molar.
Paracone	Mesiobuccal cusp, upper molar.
Metacone	Distobuccal cusp, upper molar.
Protoconid	Mesiobuccal cusp, lower molar. Unique topographical features called protostylid effects.
Hypoconid	Distobuccal cusp, lower molar.
Metaconid	Mesiolingual cusp, lower molar.
Entoconid	Distolingual cusp, lower molar.
Hypoconulid	Fifth, distal-most cusp on lower molar
Mammelons	Cusplets on incisal edges of unworn incisors.
Fissure	Cleft on the occlusal surface between cusps, dividing them into patterns such as the Y-5 pattern
Trigon/ Trigonoid	Mesial part of the upper molar/ lower molar
Talon/ Talonid	Distal part of the upper molar/ lower molar
Interproximal Contact Facets (IPCFs)	Facets formed between adjacent teeth in the same jaw, occlusal contact facets (OCFs) result from contact with of mandibular and maxillary teeth.
Cingulum	Ridge of enamel that partly or completely encircles the sides of a crown, not usually present in molars or premolars.

Particularly useful for distinguishing crown detail in Molars. Adapted from White & Folkens (2005)

1.2.5 Histological Structure and Composition of Odontological Tissues

The gross anatomy of dental tissues has been comprehensively studied as many fields of dental research are dedicated to understanding the processes involved in tooth formation, eruption, maintenance and loss, as well as anthropological facets such as age estimation, diet, microwear and disease. The histological nature of the human tooth in turn has also been extensively studied and is composed of many complex elements. White & Folkens (2005) stated that there are eighteen different features commonly found in teeth (Table 1.4). From the biomolecular perspective of this investigation however, the tooth can be roughly divided into four areas depending on dental localization, function, chemical and biological constituents; enamel, dentin, cementum and the pulp chamber (Figure 1.29).

Mature enamel is the hardest biological structure in body: varying from 5-8 on the Mohs scale of mineral hardness (where Talc = 1 Moh, Diamond = 10 Moh) (Williams & Elliot, 1989). It is a specialized hard tissue that covers the crown and is avascular and acellular. Containing less than 1% organic material, it is almost entirely inorganic and about 97% mineralized once it is formed with the rest being water (ibid). Enamel can be up to 2 mm thick over the cusps of unworn premolars and molars, and is thinner around cervical region. Enamel attains full thickness before teeth emerge into the oral cavity. The basic histological structure is calcified rods or prisms. Though the chemical composition approximates to hydroxyapatite, enamel crystallites are considerably longer than those of bone and dentin being at least 1600 ηm long. The crystallites are packed together to make a dense, crystalline mass. The mature dental enamel is almost entirely inorganic and is acellular. By dry weight fresh enamel contains 96% inorganic material, less than 1% organic material). The chemical composition of the inorganic component approximates to that of hydroxyapatite (ibid).

Dentine is not as mineralized as enamel, and therefore softer but still harder than bone. It makes up most of tooth and root and is composed of collagen and hydroxyapatite. More compressible and elastic than enamel, it is less likely to crack or fracture. Dentine is a mineral-organic composite. It contains 72% inorganic material by dry weight, 18% collagen and 2% other organic material (Williams & Elliot, 1989). The majority of the mineral is apatite, with crystallites much shorter than those in enamel at 20-100 ηm in length. Some amphorous calcium phosphates may also be present. One of the dominant features of dentine structure is the collagen, which is secreted in mats of fine fibres. Unlike enamel dentine is a living tissue and the cells of dentine (odontoblasts) line the sides of the pulp chamber.

Once dentin has been formed, it does not ‘turn over’, unlike bone in which the cells are constantly replaced. Secondary dentine formation continues after the initial formation on the walls and roof of the pulp chamber (Hillson, 2001).

Cementum covers the roots of the teeth, and is a bone-like rigid connective tissue located over the dentine, with a thickness varying from 100-200 μm to 500 μm depending on the location and age of the individual. Its main function is to attach the periodontal ligament to the surface of the tooth. The cement has no nervous or blood supply and the cells that compose it are found at the interface or within the richly supplied collagen fibers of the ligament that attaches the tooth cement to the alveolar bone of the jaw. Cementum has a mineral to organic ratio comparable to bone, though as an avascular tissue it exhibits little to no remodelling, and continues to grow throughout life (Freeman, 1994; Grant et al., 1988; Saygin et al., 2000).

Several distinct varieties of cementum occur in teeth. These are defined according to the presence or absence of cells, source of collagen fibres, rate of formation, chemical composition and degree of mineralization. Three different varieties of cementum can easily be identified; acellular afibrillar cementum, acellular extrinsinc fiber cementum and cellular extrinsic cementum (Bosshardt & Selvig, 1997).

The pulp chamber is an extended part of the pulp cavity at the crown end of the tooth which serves to support odontoblast dentin cells, nerve trunks and blood vessels. It can be divided roughly into three layers, a cell rich innermost layer containing fibroblasts and undifferentiated mesenchymal cells; a cell free zone (or zone of Weil) which is rich in vascular tissue such as capillary and nerve networks (nerve plexus of Rashkow), and the outer layer or odontoblastic layer, which contains the odontoblasts and lies next to the predentin and mature dentin. The cells found in the dental pulp include fibroblasts, odontoblasts and defense cells such as histiocytes, macrophages, granulocytes, mast cells and plasma cells (Hillson, 2002).

Table 1.4: Permanent Dentition Eruption Schedule in Humans				
Initial Calcification	**Crown Completed**	**Typical Eruption Age Range**	**Eruption Sequence**	
			Mandibular	**Maxillary**
Birth	2.5-3 Years	6-7 Years	First Molars	First Molars
3-4 Months	4-5 Years	6-7 Years	Central Incisors	
3-4 Months	4-5 Years	7-8 Years		Central Incisors
3-4 Months	4-5 Years	7-8 Years	Lateral Incisors	
10-12 months	4-5 Years	8-9 Years		Lateral Incisors
4-5 Months	6-7 Years	9-10 Years	Canines	
1.5-2 Years	5-6 Years	10-12 Years	First Premolar	
1.5-1.75 Years	5-6 Years	10-11 Years		First Premolar
2-2.25 Years	6-7 Years	10-12 Years		Second Premolar
2-2.25 Years	6-7 Years	11-12 Years	Second Premolar	
4-5 Months	6-7 Years	11-12 Years		Canine
2.5-3 Years	7-8 Years	11-13 Years	Second Molar	
2.5-3 Years	7-8 Years	12-13 Years		Second Molar
7-9 Years	12-16 Years	17-21 Years	Third Molar	Third Molar

In general, the eruption sequence is mandibular before maxillary. Active eruption occurs after one-half of the root is formed. Apex is fully developed 2-3 years after the eruption. With regards to this research the majority of the dental samples taken for analysis consisted of third, second and first molars in that order, preference given where possible to mandibular samples (Highlighted). Adapted from Ash & Nelson, 2003.

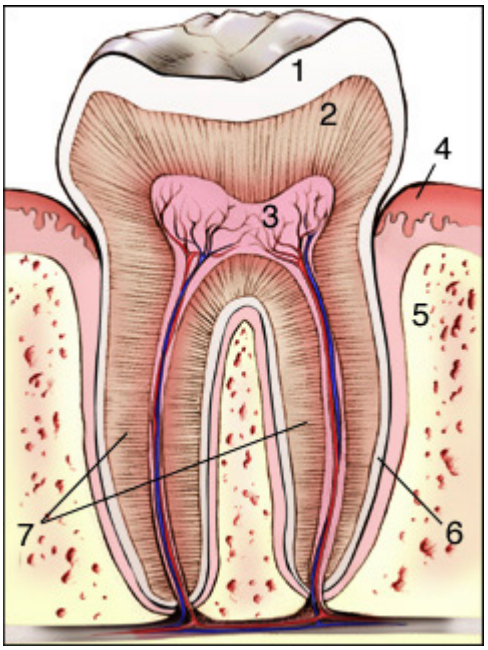


Figure 1.29 Longitudinal Section Schematic of the Typical Human Tooth. Diagram displays predominant tissue types (enumerated) found in human teeth, in this case exemplified by a Molar type tooth.
1. Enamel
2. Dentine
3. Pulp Chamber
4. Gingiva
5. Periodontal Ligament
6. Cementum
7. Root (Root Canals)

Figure from www.healthopedia.com.

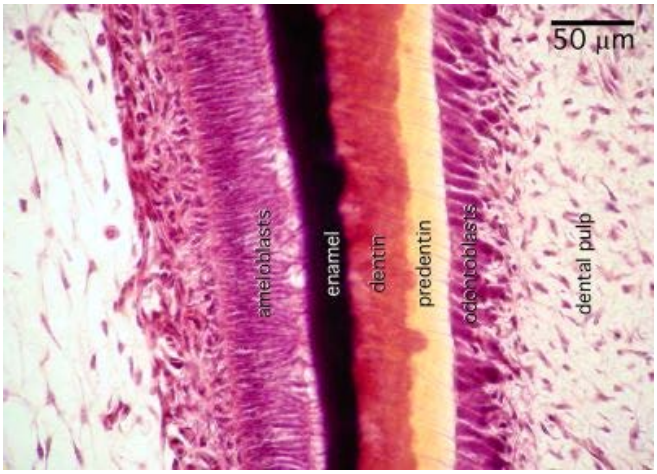


Figure 1.30 Hematoxylin & Eosin Stained Longitudinal Tooth Section. Shows the dentin pulp chamber interface and the interaction between the dentin tubules and the odontoblasts at the dentin matrix with the cell rich zone of the pulp chamber. (Pig tooth, 400 x magnification). Courtesy of William L. Todt.

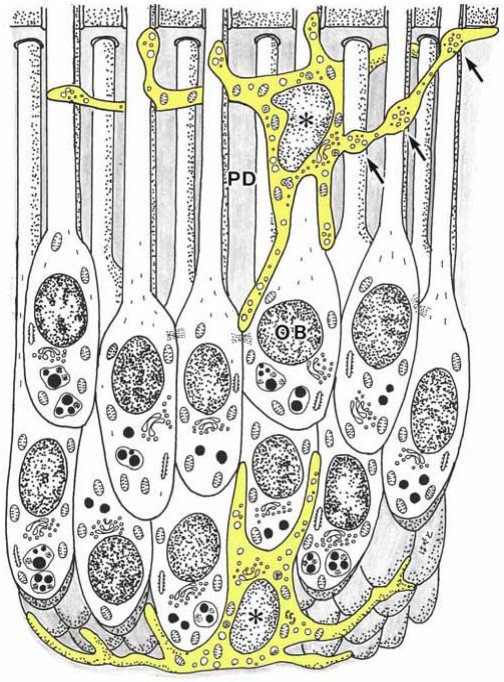


Figure 1.31 Schematic of Dentin Pulp Junction. From this illustration the interface between the odontoblast cells (OB), emerging from dentinal and pre-dentinal (PD) tubules into the nutrient rich pulp chamber can be clearly seen. It is believed that the resorption of this interface into the dentinal tubules and subsequent calcification the source of the high quality DNA in dental remains. Figure from www.dent.niigata-u.ac.jp.

1.2.6 Dental Cytology; Distribution & Function

Ameloblasts are not found in developed teeth, and hence are of limited interest to the anthropologist. The ameloblasts play a key role in the development of the tooth cap, where they secrete enamel proteins and amelogenin which later mineralises into enamel. An hexagonal cell of 4 μm in diameter and 40 μm in length, ameloblasts only become active after the first layer of dentin is formed (Hillson, 2002).

Odontoblasts are primarily found in the dentin, but also found along with cementoblasts in the periodontal ligament. Odontoblasts are long cylindrical cells varying between 4-7 μm in diameter and are responsible for secreting the initial pre-dentin matrix to produce mature dentin (Figure 1.30). At the end of the odontoblast cell is a fine process that tapers into a dentinal tubule that occasionally bears offshoots to interconnect with other processes. The odontoblast processes occupy and create the dentinal tubules, which may extend the amount of dentin when required. The dentinal tubules radiate out from the pulp chamber, primarily along an S-shaped course curving from the apical to the occlusal, with a secondary corkscrew curvature. The odontoblast cells and process passes through the full thickness of dentin and remains throughout the life of the tissue, one tubule per cell (Figure 1.31) (ibid).

Odontoclasts are large cells of 50 μm or more in diameter, which resorb cement and dentin from the root surface and are associated with irregular depressions cut into the cement or dentin surface. These depressions, known as resorption hollows or howship lacunae, are highly variable in size and their scalloped edges are representative of odontoclast activity.

Fibroblasts and fibrocytes are cells most commonly found in connective tissues, where they synthesise the basic structural framework (or stroma) for animal tissues, the extra cellular matrix and collagen). Fibroblasts and fibrocytes being essentially the same cell at different state of metabolism (as with osteoblasts and osteocytes (§ 1.2.3)). With regards to teeth, these cells control the rapid turnover of the periodontal ligament by a highly active secretion and resorption of the collagen fibres (ibid).

Cementoblasts are variable in size shape and orientation, from 5-17 μm and present numerous cell processes radiating from their cell bodies, seen as small thread like tendrils that extend towards the cement. These fine processes that extend on all sides, average 12-49 μm in length with 8-20 processes per cell (Scivetti, 1996). These cell processes extend through a system of interconnecting canaliculi and anastomase with the neighbouring cementocytes. They are contained in irregular and variable spaces that are called cementocyte lacunae. This

system may be responsible in maintaining the nutritional supply to the entrapped cellular component (MacNeil & Somerman, 1993; Bosshardt & Selvig, 1997). Cementoblasts themselves are responsible for remodelling the cementum in response to the rapid turnover of the interconnecting collagen fibres of the periodontal ligament where they are located.

Cementoblasts are cells that have been surrounded by the developing pre-cement matrix. The variability of cell size and the number of processes per cell is believed to be related to the nutritional status and the depth within the cementum matrix (Bosshardt & Selvig, 1997) since the processes need to reach to the surface of the cementum to draw nutrition from the periodontal ligaments. The small processes run along channels called canaliculi that run irregularly through the cement, occasionally connecting with lacunae. Invariably the further the cementocyte is within the cement, and thus further from the surface, the least active the cell is.

THE CHEMISTRY OF FOSSILISED REMAINS

1.3 Hard Tissues; Matrix and Chemistry

The intracellular matrix of hard tissues is made up of a hard ground substance simply defined as a non-living matrix of connective tissue (Lawrence, 2005), interstitial fluid, free electrolytes and connective tissue fibres. In skeletal remains, the hard tissue matrix can be divided into two factions, an inorganic or mineral faction consisting of biogenic apatites of calcium phosphate and an organic faction consisting predominantly of collagenous and non-collagenous proteins. The combination of both factions gives bone its unique characteristics of rigidity and flexibility. In archaeological specimens, where bone has been demineralised (normally due to extreme pH or chelating agents), it becomes a soft pliable entity of collagenous fibres, and, where the organic collagen has been removed (either by cremation, biological breakdown or reverse leaching) the bone becomes extremely brittle and crumbles easily. The following sections provide more detailed information about the organic and inorganic factors of teeth and bones.

1.3.1 The Organic Faction of Hard Tissues

A significant portion of the organic component of dentine, cement and bone is protein (Table 1.5). Fresh compact bone consists of 20% protein by weight (wet or dry) (Hedges & Millard, 1995), of which approximately 65% is collagen (Puzas, 1993). Collagen fibrils are visible under polarized light as mature cross-linked fibres. These fibrils, with diameters of up to 3000Å, are polymerised from hollow tubes of` five microfibrils each 44Å in circumference formed from the basic collagen unit, the tropocollagen subunit (c. 3000x16Å) (Mathews, 1980). The collagen molecule is 280-300 ηm long and made up of three α-chains each consisting of >1000 linked amino acids residues with a combined molecular weight of 100 kDaltons. There are four types of α-chain distinguished by minor difference in amino acid sequence but in bone, dentine and cementum, all are type 1 chains. In bone, type 1 collagen makes up 85-90% of all the collagen present (Termine, 1993). These chains are built from 20 amino acids, but glycine compromises one third of the amino acid residues and there are about 10% each of proline and its derivative hydroxyproline.

Table 1.5: Comparison of the Composition of Mineralized Tissues.

Component	Enamel		Dentine		Bone[b]
	Weight	Volume	Weight	Volume	Weight
Inorganic %	>96	88 (80-100)	72	50	70
Organic %	<0.2-0.6	0.3	20 (18[c])	30	22 (21[c])
Density g/cm^3	2.9-3	N/A	2-2.3	N/A	2-2.05
Calcium %	34-40		26-28		24
Phosphorus %	16-18		12.2-13.2		11.2
Ca/P ratio (weight)	1.92-2.17		2.1-2.2		2.15
Ca/P ratio (molar)[a]	1.5-1.68		1.6-1.7		1.66
CO_2 as carbonate %	1.95-3.66		3-3.5		3.9
Sodium %	0.25-0.9		0.7		0.5
Magnesium %	0.25-0.56		0.8-1		0.3
Flourine ppm	<25-5000		50-10000		5000
Iron ppm	8-218		60-150		N/D
Zinc ppm	152-227		200-700		N/D
Strontium ppm	50-400		100-600		N/D

[a]Compared Ca/P ratio (molar) 1.667 for pure hydroxyapatite; [b]Cementum analysis not available but similar to bone, [c] %Collagen. Adapted from Hillson, 1996. Sources Brudevold & Soremark, 1967; Rowles, 1967; Williams & Elliot, 1989.

Table 1.6: Amino Acid Composition of Fresh Enamel, Dentine and Bone

Amino Acid	Enamel[1, 4]	Dentine[1, 4]	Bone[1, 5]
Cysteine	0.6	0.01	N/A
Arginine	7.0	8.5	8.8
Histidine	0.56	0.54	0.96
Lysine	2.5	2.5	4.40
Aspartic	5.2	7.0	6.7
Glutamic	10.1	10.6	11.4
Glycine	19.7	17.4	25.8
Alanine	9.6	6.9	10.9
Serine	6.4	2.7	3.5
Threonine	4.3	2.2	4.06
Valine	3.4	2.5	2.97
PLI[2]	13	9.4	2.49, 3.60, 1.88=7.97
Methionine	1.0	0.62	0.85
Tyrosine	1.0	1.1	0.86
Proline	4.0	5.2	15.3
Hydroxyproline	5.0	16.2	14.1
Tryptophane[3]	0.10	1.1	N/A

All values given as % of dry weight; [2]Phenylalanine, leucine & isoleucine; [3]Probable value for tryptophane. Adapted from [4]Barristone & Burnett, 1956; [5]Eastoe, 1955.

Around 10-15% of the protein of bone is made up of Non-Collagenous Proteins (NCPs), a quarter of which are believed to be from exogenous origins, absorbed or trapped within the bone matrix (Termine, 1993). The function of the majority of these NCPs is not well understood, although the two most common, osteocalcin and osteonectin, show a high affinity for the inorganic components of bone, and osteonectin is particularly associated with bone turnover (Termine, 1993). Additionally, the cell attachment proteins, such as fibronectin, thrombospodin, osteopontin and sialoprotein, are associated with the control of extracellular macromolecules (Termine, 1993). Glycoproteins and proteoglycans are highly anionic complexes important for mineralisation (Baron, 1993), and proteoglycans are associated with the synthesis of collagen fibrils (Stanford, 1993). An interesting observation regarding NCPs, is that, on a mole for mole basis, it is estimated that half of the biosynthetic activity of the osteoblast is concerned with the production of NCPs (Termine, 1993).

When considering the organic faction at the amino acid level, the characteristic amino acid composition of the human bone resembles that of pure collagen, which contains 33% glycine, 9% hydroxyproline, 11% proline 7% aspartic acid, 9% glutamic acid, 5% hydroxylisane and trace concentrations of the remaining 12 amino acids (Table 1.6). Survival of the proteins is highly variable but a bone may be considered well preserved when it has 5% or more dry weight of protein, in contrast to the >20% in fresh tissue (Schwarcz & Schoeniger, 1991).

The protein content in archaeological dentine shows little relationship with archaeological age, but bears some relationship with the structural integrity of dentin (Beeley & Lunt, 1980). It has been observed that softer specimens may have only 0.1% -8% protein compared to 11-19% protein by dry weight in harder specimens. As with bone the great majority of the protein in the tooth in collagen type 1. In dentin the proteoglycans are the largest non-collagen faction, they are large molecules, with a protein core linked to carbohydrate chains (glycosaminoclyans or acid mucopolysaccharides). Other protein components of dentin include phosphoproteins, proteins containing γ-carboxyglutamic acid (called osteocalcin in bone) and proteins normally found in serum, including albumin. As with bone, dentine contains a small component of lipids and other smaller organic molecules including citrate and lactate. As specified in table 1.5, the majority of enamel is inorganic. Only around 1% of enamel is believed to be of organic origin, and of this 90% is considered to be amelogenin or a breakdown product of amelogenin. Amelogenin molecules exist in a variety of sizes, derived from the largest form, which has a molecular weight of 20 kD. Amelogenin is a highly unusual protein, with proline making up a quarter of its amino acid residues, followed

by glutamic acid, leucine and histidine. Compared to collagen, hydroxyproline is conspicuous by its absence, which is useful in identification amongst other sources of amino acids. During the maturation process in enamel, rapid protein removal occurs through a cascade of amelogenin degradation in which enzymes cleave the protein, leading to low levels of this protein in the mature enamel (Hillson, 2002).

1.3.2 Inorganic Faction of Hard Tissues

The mineral component (or inorganic faction) is interspersed among the collagen fibres giving both compressional strength and hardness to the bone matrix. Living calcified tissues contain between 69% and 99% (by weight) of inorganic material (Table 1.5). In all the hard tissues, this inorganic component consists almost entirely of calcium phosphate minerals, mostly in the form of apatite. Calcium phosphates comprise the largest group of biominerals in vertebrate animals. The phosphates containing HPO_4^{2-} and PO_4^{3-} generally constitute the biologically relevant calcium phosphates (Table 1.7). Apatites are compounds of the general formula $M_{10}(XO_4)_6Y_2$, rather than for specific compounds. In general they are known to accommodate a variety of modifications and substitutions of ions (Y^-), cations (M^{2+}) and groups (XO_4^-) within the apatitic lattice. The term apatite has become synonymous with calcium phosphates: $Ca_{10}(PO_4)_6X_2$ where X can be a variety of ions, the most common being hydroxyl (OH^-) flouride (F^-) and chloride (Cl^-). Apatites are thermodynamically the most stable among the calcium phosphates and therefore can be considered the end product in many reactions. Calcium and phosphate may also be replaced by other ions, and there is a continuous variation from one to another, with partial or complete substitution of any ions in the general formula (Elliot, 1997).

In geology, apatites, although not abundant, occur most often in a form similar to fluoroapatite ($Ca_{10}(PO_4)_6F_2$), which is the most stable structure among the apatites (Sudarsanan, et al., 1972). In biological tissues, however the predominant form is the crystalline hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (Baron, 1993), which is found predominantly in biological tissues. Pure crystalline hydroxyapatite is considered more of a model structure as it is never in a pure state. Crystalline hydroxyapatite incorporates a wide variety of impurities and is seldom found in pure stoichiometric form. The most common substitutions are with calcium (Ca^{2+}), strontium (Sr^{2+}), phosphate (PO_4^{3-}), flourine (F^-), carbonate (CO_3^{2-}) and hydroxide (OH^-) ions (Pate & Hutton, 1988). The favoured crystalline structure is hexagonal as described by Kay et al., (1964) and depicted in figure 1.32.

Table 1.7: Calcium Phosphates and Associated Structures in Biological Tissues		
Calcium Phosphate	**Molecular Name**	**Molecular Formula**
Apatite Type $M_{10}(PO_4)_6X_2$	Hydroxyapatite (OHAp)	$Ca_{10}(PO_4)_6(OH)_2$
	Flourapatite (FAp)	$Ca_{10}(PO_4)_6F_2$,
	Chlorapatite (CLAp)	$Ca_{10}(PO_4)_6Cl_2$
Apatite related	Octacalcium phosphate (OCP)	$Ca_8(HPO_4)_2(PO_4)_4.5H_2O$
	Tetracalcium phosphate (TTCP)	$Ca_4(PO_4)_2O$.
Glaserite Type	Tricalcium Phosphates (TCP)	$Ca_3(PO_4)_2$
Ca-PO_4 Sheet containing compounds	Dicalcium phosphate dihydrate (DCPD)	$Ca_2(HPO_4)_2.2H_2O$
	Dicalcium phosphate anhydrous (DCPA)	$Ca_2(HPO_4)_2$
	Monocalcium phosphates	(MCPM) $Ca(H_2PO_4)_2.H_2O$ (MCPA) $Ca(H_2PO_4)_2$.
Struvite-type structures	Magnesium sodium phosphate heptahydrate	$Mg(NH_4)PO_4.6H_2O$
Amorphous calcium phosphates (ACPs)		$Ca_3(PO_4)_2.nH_2O$

Examples of some of the various forms of calcium phosphates actually found in the inorganic structure of bones & teeth. Table adapted from information from Mathew & Takagi, 2001.

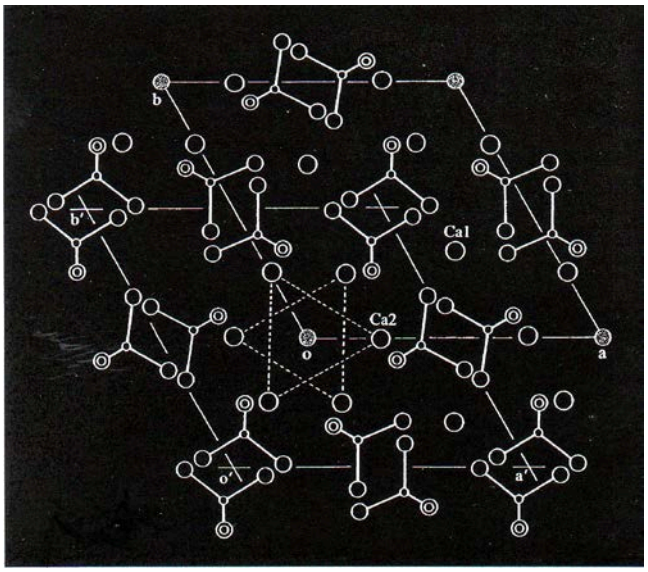


Figure 1.32: Structure of Crystalline Hydroxyapatite in Hard Tissues.
Figure from Mathew & Takagi, 2001.

A large number of proposals have been put forward to account for the nonstoichiometry of bioapatites. Various impurities are incorporated, usually substitutionally for calcium, but also interstitially magnesium, strontium and lead. These are commonly described as the bone seeker as they accumulate in bone and tooth minerals where the incorporation of foreign cations in the apatite lattice changes the bulk properties of the apatite. In a lead apatite study a short Pb-O distance was observed indicating that a covalent bond maybe involved in lead accumulation (Mathew et al., 1980). Both HPO_4^{2-} and structurally incorporated water also occur in biological apatites, but their structural locations are unknown. Apatites are of special interest in biological systems as the inorganic component of hard tissues and there are two generally accepted locations for the CO_3^{2-} ion in the apatite lattice. Carbonate apatites are principally type-B carbonates, but there are also small amounts of type-A (Elliot et al., 1985). However, neither of these cases has been confirmed by complete structural analysis and the structure of carbonate-apatite remains undetermined. The mineral structure, as is an amphorous calcium phosphate structure, incorporating not just traditional apatite structures, but those of octacalcium phosphates and other possible calcium phosphates precursor structures and possibly struvite-type structures as precursors to produce a dynamic and variable crystal structure (Table 1.7).

The calcium concentration in bone (0.5 mmol/L) is lower than in plasma (1.5 mmol/L) suggesting a constant flow of calcium out of the bone (Baron, 1993). Hence, maintenance of the mineral homeostasis is an important property and one of the most significant features is the ability of calcium phosphate to exist in various forms, from amphorous to crystalline (Sillen, 1989). Bone mineral therefore has a spectrum of intermediates seen in the transformation of a solution of Ca^{2+} and P^{3+} ions to solid hydroxapatite (Neuman, 1980). Hydroxyapatite itself being a highly variable structure particularly in bone and cementum where it is in continuous flux. The most common form or model form of the hydroxyapatite of calcium phosphate in bone is $Ca_{10}(PO_4)_6(OH)_2$. Crystal rods are approximately 30-50 Å in diameter and up to 600 Å in length (Puzas, 1993) and orientated in parallel with the closely associated collagen fibres.

Apart from the bioapatites and their elemental constituents, the inorganic faction is also made up of many other trace elements, some of which are deemed essential to human life and others non-essential, although there are those whose role remains ill defined, such as in the case of cobalt. Twenty five of which are recognized as essential to human life. Of the essential elements, the human body is predominantly composed of the six major elements,

oxygen, carbon, hydrogen, nitrogen, calcium and phosphorus that make up over 99% of the human body in the following ratios 61% oxygen, 23% carbon, 10% hydrogen and 2.6 % nitrogen, 1.4% calcium and 1.10% phosphorus. Consequently, there are six minor elements that also play are also considered important role although the make up a lower percentage of the total human body the concentration of which can be determined by grams per kilogram. These elements and their ratios are sulphur 0.2 %, potassium 0.2%, sodium 1.4 %, chlorine 1.2%, magnesium 0.03% and silicon 0.03%. The remaining 71 elements of the periodic table can be considered, as an expression of their low concentration in the human body, as "trace elements", however, in most cases there is a far smaller subset of elements that are commonly found in the body at detectable concentrations of between 0.01 and 100 ppm (Curzon & Cutress, 1983). The following section deals with them in more detail.

1.3.3 The Trace Elements of the Inorganic Faction of Hard Tissues

A list of the known trace elements present in calcified tissues and estimated normal concentrations is given in table 1.8. Trace elements are notable not just for there low concentrations, but their important role in human health, which is reflected in the acute sensitivity of the body to some of these elements. In regard to both excessive concentrations that can be toxic, and scarce concentrations that can cause dietary deficiency and subsequent adverse effects. Magnesium, due to its dietary importance, will for the purposes of this study, be considered as part of the trace elements, despite its higher concentration in the human body (Curazon & Cutress, 1983).

Trace elements, in particular the essential ones, are generally thought to have two general functions in the human body; as a structural material and as regulator of the numerous biological activities of the body (Aras & Ataman, 2006). A summary of the significance of several trace elements in calcified tissues, in particular teeth, and there expected concentrations both in the human body and in enamel are given in table 1.9 and the dietary importance of specific elements with regards to this thesis are considered. The predominate mode of uptake in humans is through ingestion as a component of food and water, although other mechanisms have been shown, in particular with teeth, where direct absorption can take place after prolonged periods. Several models of trace element dynamics in the body have been put forward, most involving a series of deposition and release cycles that are dependent on the structural modelling and remodelling of the calcified tissue and the metabolic

requirements, a simplified model of the dynamics of the uptake of trace elements is given in figure 1.33.

Table 1.8: Trace Elements & Normal Concentrations in Human Tooth Enamel

Symbols	Elements	Concentrations*	Symbols	Elements	Concentrations*
^{40}Ca	Calcium	>1000	^{86}Rb	Rubidium	1.0-10
^{30}P	Phosphorus	>1000	^{80}Br	Bromine	1.0-10
^{18}F	Flourine	>1000	^{48}Ti	Titanium	1.0-10
^{24}Mg	Magnesium	>1000	^{79}Se	Selenium	0.1-0.9
^{22}Na	Sodium	>1000	^{59}Ni	Nickel	0.1-0.9
^{35}Cl	Chlorine	>1000	^{56}Co	Cobalt	0.1-0.9
^{39}K	Potassium	100-1000	^{7}Li	Lithium	0.1-0.9
^{32}S	Sulphur	100-1000	^{107}Ag	Silver	0.1-0.9
^{65}Zn	Zinc	100-1000	^{92}Nb	Niobium	0.1-0.9
^{28}Si	Silicon	100-1000	^{9}Be	Beryllium	0.1-0.9
^{88}Sr	Strontium	100-1000	^{91}Zr	Zirconium	0.1-0.9
^{11}B	Boron	10-100	^{183}W	Tungsten	0.1-0.9
^{56}Fe	Iron	10-100	^{121}Sb	Antimony	0.1-0.9
^{27}Al	Aluminium	10-100	^{50}V	Vanadium	<0.1
^{137}Ba	Barium	10-100	^{74}As	Arsenic	<0.1
^{208}Pb	Lead	10-100	^{132}Cs	Caesium	<0.1
^{64}Cu	Copper	1.0-10	^{243}Am	Americium	<0.1
^{52}Cr	Chromium	1.0-10	^{138}La	Lanthanum	<0.1
^{96}Mo	Molybdenum	1.0-10	^{72}Ge	Germanium	<0.1
^{55}Mn	Manganese	1.0-10	^{141}Pr	Praseodymium	<0.1
^{112}Cd	Cadmium	1.0-10	^{144}Nd	Neodymium	<0.1
^{118}Sn	Tin	1.0-10	^{158}Tb	Terbium	<0.1
^{127}I	Iodine	1.0-10	^{88}Y	Yttrium	<0.1

List of commonly found elements in human tooth enamel, including predominante trace elements and normal range of concentrations found in healthy modern Individuals. * Concentrations given in ppm. Adapted from Curazon & Cutress, 1983.

Table 1.9: Estimated Concentrations and Functions of the Predominate Trace Elements Found in the Human Body and Human Tooth Enamel			
Elements	**Concentrations in human body***	**Concentrations in human enamel***	**Functions**
Magnesium	N/A	> 1000	Roles in bone mineralization, protein synthesis, enzyme action, muscular contraction, nerve transmission
Iodine	N/A	1 - 10	Part of thyroxin, which regulates metabolism
Iron	100	10 - 100	Haemoglobin formation, part of myoglobin, energy utilization
Zinc	100	100 - 1000	Part of many enzymes, present in insulin, involved in making genetic material and proteins, immunity, vitamin A transport, taste, wound healing, making sperm, normal fetal development
Copper	10	1 - 10	Absorption of iron, part of several enzymes
Fluoride	100	N/A	Formation of bones & teeth, helps make teeth resistant to decay & bones resistant to mineral loss
Selenium	N/A	0.1 - 0.9	Helps protect body from oxidation. No evidence adverse affects from either excess or deficiency.
Chromium	0.1	1 - 10	Associated with insulin and required for the release of energy from glucose
Molybdenum	0.1	1 - 10	Facilities enzyme functions and many cell processes
Manganese	1.0	1 - 10	Facilities enzyme functions and many cell processes
Cobalt	0.1	0.1 - 0.9	Part of vitamin B12, which involves in nerve function and blood formation
Vanadium	N/A	< 0.1	Control of sodium pump: inhibition of ATPase, *p*-transferases
Nickel	0.1	0.1 - 0.9	Constituent of urease, reduced haemopoiesis
Cadmium	1.0	1 - 10	Stimulates elongation Betois in ribosomes
Tin	1.0	1 - 10	Interactions with riboflavin
Lead	10	10 - 100	Many enzyme effects
Lithium	N/A	0.1 - 0.9	Control of sodium pump
Arsenic	N/A	< 0.1	Increased arginine urea + ornithine, Meto, metabolism of methyl compounds
Boron	N/A	10 - 100	Control of membrane function, nucleic acid biosynthesis and lignin biosynthesis
Strontium	10	100 - 1000	No determined role

Comparison of expected concentrations of trace elements in the human body compared to uptake by human enamel. Functions are outlined, for indication of purpose, importance for nutrient uptake and element sequestration and possible association to tooth concentrations. Data from Curzon & Cutress,1983 and Talwar & Srivastava (2004).*Concentrations given in ppm, N/A – Not Ascertained,

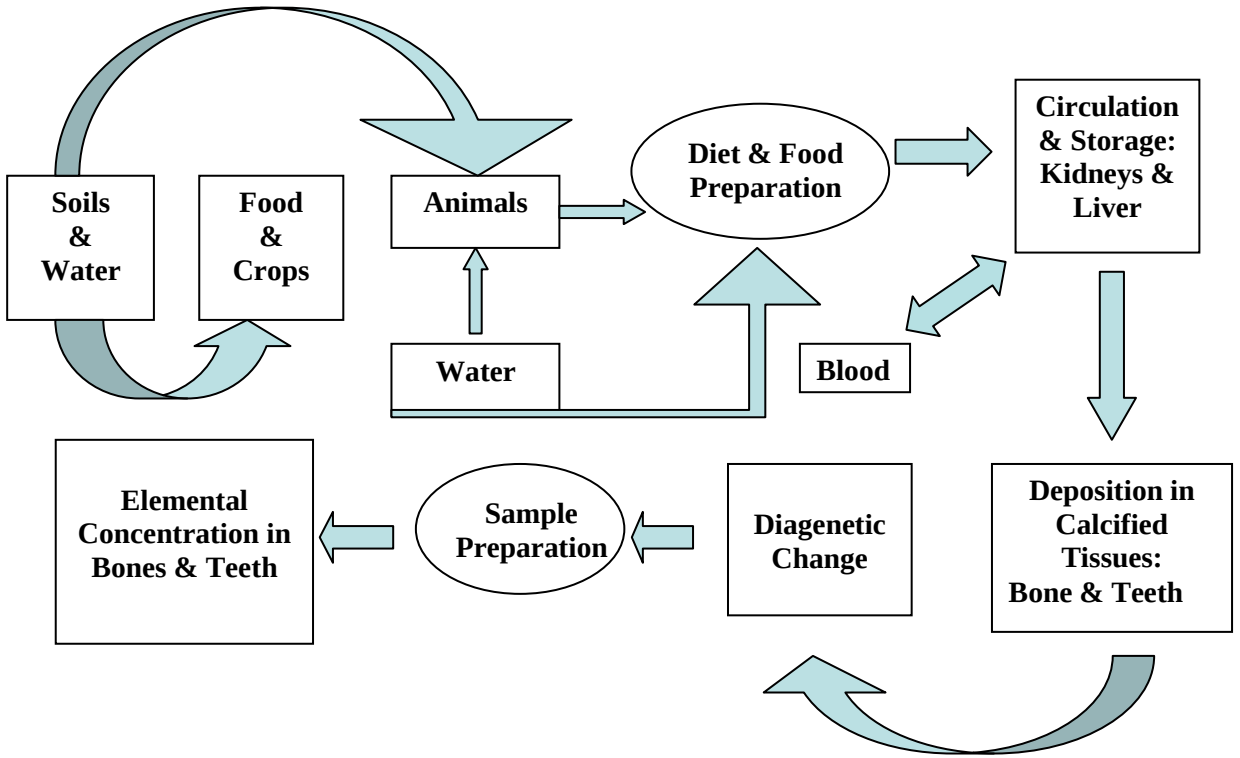


Figure 1.33: A Flow Diagram showing Simple Biological Uptake and Deposition Model for Elements in Bone and Teeth

Magnesium (^{24}Mg): One of the most abundant elements in the human body, at about 24 grams, 60% of which is sequestered into the skeleton. It plays a major role in human physiology, is the fourth most abundant element and has a diverse and ubiquitous range of physiological functions (Arnaud, 2008), including pivotal role in the functional of activity of an estimated 300 or more enzymes. It has roles in bone mineralization, DNA and protein synthesis, enzyme action, normal muscular contraction and nerve transmission (Aras & Ataman, 2006). Microbial action has been shown to reduce magnesium concentration in calcified tissues (Grupe & Piepenbrink, 1989). There is an inverse relationship between body mass index and magnesium concentration (Wang et al., 2005). Alcoholism in particular can cause magnesium depletion. Severe deficiency of magnesium has been linked to cystic fibrosis (Chatt & Katz, 1988) to neurological disorders (Lech, 2002). Excess of magnesium or hypermagnesemia, particularly in blood, is controlled by the kidneys, so dietary toxicity is unusual, but can occur in individuals with poor renal function, although it has been reported usually related to high cathardic doses of magnesium salts (Kontai et al., 2005).

Manganese (^{55}Mn): An essential trace mineral at low concentrations, it functions as a cofactor in a number of enzymes and many cell processes (Aras & Ataman, 2006), particularly the detoxification of free radicals. In this role it influences glucose and lipid metabolism, connective and calcified tissue formation, growth, reproduction and the immune system. In humans, there are an estimated 12 -20 mg of manganese. This is usually concentrated the liver and kidneys, although it has a tendency to accumulate in dental tissues, at levels of around 3.5 ppm (Lane & Peach, 1997). Manganese deficiency or excess can cause serious clinical effects (Kamberi, 2009). A manganese deficiency is very rare in humans and does not usually develop unless manganese is deliberately eliminated from the diet. Deficiency has been linked to chrondrogenesis and skeletal defects. Although manganese is less toxic than other abundant trace metals such as nickel and copper, excess has been linked to impaired motor skills and cognitive disorders. Excessive prenatal exposure has been linked to behavioural abnormalities in later life and highly toxic levels of manganese lead to particular a form of neurodegeneration called manganism. Manganism shares a number of similarities with Parkinson's disease, and causes impotency, hallucinations, violent acts and irritability (Normandin & Hazel, 2002). Manganism has been predominantly found in industrial workers, particularly miners, exposed to manganese dust, since the 19th century (Crossgrove & Zheng, 2004). Toxic levels of manganese are also associated with chronic liver disease, as the liver plays an important role in eliminating excess manganese from the body.

With regards to dental material, excess manganese has been shown to cause amelogenesis (Elwood 1962)., and in archaeological material, elevated levels of manganese have been detected in black stained enamel (Stermer et al., 1996) and appear to have a correlation with aluminium levels (Pate & Hutton, 1988).

Iron (^{56}Fe): Iron is an essential mineral in nearly all organisms. In humans it is utilized in the heme complex, which in turn is an essential part of the cytochrome proteins that mediate redox reactions, and the oxygen carrier proteins hemoglobin, myoglobin and leghemoglobin (Aras & Ataman, 2006). Iron distribution in mammals is heavily regulated, possibly due to both the high potential for toxicity (Nanami et al., 2005) and to inhibit microbial infection. Lack of iron can ultimately cause iron deficiency anaemia. Iron deficiency can result in weakness, fatigue, paleness of the skin and constipation, predominantly due to oxygen starvation and muscular fatigue. Usually associated with genetic defects in genes associated with iron regulation (HLA-H gene region on chromosome 6), where an excessive iron intake can cause disorder such as haemochromatosis. High levels of unbound iron, particularly in cells, is believed to catalyse the production of free radicals from peroxidase. Iron toxicity therefore can affect a number of tissues and consequently lead to a variety of clinical effects, including coma, metabolic acidosis, liver failure, coagulpathy, respiratory distress and even death (Cheney et al., 1995).

Cadmium (^{112}Cd): The specific biological enzyme carbonic anhydrase has cadmium as a reactive centre and cadmium may also stimulate the elongation in ribosomes (Ares & Ataman, 2006). The uptake and use of cadmium is intimately related to zinc, as the two elements are chemically similar, cadmium can substitute for zinc in many biological systems, including the zinc binding proteins and can bind up to ten times more strongly. It is estimated that 30 mg of cadmium in naturally found in humans, the far greatest concentration is found in the kidneys (10 mg) and the liver (4 mg). The metabolism of cadmium is influenced by the uptakes of both copper and zinc. Generally, cadmium is considered a toxic compound even at low concentrations, and is known to accumulate biological systems (Talwar & Srivastava, 2004). Deficiency of cadmium has no known adverse effects. Cadmium is considered a carcinogen. Elevated levels may reflect systematic intoxication (Chatt & Katz, 1988) is believed to cause decreased fertility, pneumonitis, pulmonary edema and death. Tobacco smoking is currently the greatest source of cadmium exposure, although specific intoxication is normally associated with industrial processes, and absorbed trough inhalation. Excess cadmium causes effects similar to iron deficiency, and may be a causal factor of hypertension in human.

Zinc (^{66}Zn): An essential component in humans (Prasad, 2008) and is found in over 100 specific enzymes and is involved in the synthesis and control of carbohydrates, lipids, proteins and nucleic acids as well as the metabolism of other trace elements with a central role in the immune system (Shanker & Prasad, 1998; Cotton et al., 1999).

Zinc concentrations are associated with elemental levels in soil, particularly in plants (Broadley et al., 2007). Deficiency is normally related to dietary intake or malabsorption and can be associated with a variety of disorders including acrodermatitis, enteropathica, chronic liver disease, chronic renal disease, sickle cell disease and diabetes. Zinc deficiency may result in poor growth, acne-like rash, hair loss, diarrhoea, delayed sexual maturation, impotence, sterility, eye lesions, loss of appetite, reduced sense of taste and smell, skin lesions and inflammation, poor wound healing, impaired immunity to infection, mental confusion, poor learning ability, changes in hair and nails and anaemia (Prasad, 2003). An excess of zinc is rare but possible, and can also lead to a variety of clinical outcomes including diarrhoea, cramps, nausea, vomiting, suppressed immune function, impaired formation of red blood cells and reduced levels of high density lipoprotein (HDL) cholesterol (Rink & Gabriel, 2000). From a trace element perspective, excess zinc can lead to the suppression of copper and iron uptake (Fossmire, 1990). Post burial zinc has been shown to rapidly exchange with calcium on the apatite crystal surface in the environment (Samachson, 1967).

Copper (^{64}Cu): Copper in the human body affects the adsorption, storage and metabolism of iron and is part of several enzymes (Aras & Ataman, 2006). With an estimated 60-100 mg in the body, its only next to iron and zinc in bioconcentration in humans. Essential for red blood cells (RBT), connective tissue & transmission of nerve impulses, and immune system regulation it is readily incorporated in teeth. The type of diet can affect the net adsorption of copper in the diet, phylate, ascorbic acid, zinc and cadmium amongst other heavy elements can impair the uptake of copper. Copper deficiency has been associated with a wide variety of disorders, including depressed growth, bone disorders, anaemia's, neonatal ataxia, impaired reproduction gastro-intestinal disease, cardivascular defect and can cause a reduction of the white blood cell count (WBT) (Talwar & Srivastava, 2004). Copper toxicity is extremely rare, though it when it occurs is normally associated with liver damage, and related to genetic disorders impairing liver function such as Wilson's disease.

Strontium (^{88}Sr): Strontium is believed to stimulate osteoblast proliferation and bone formation (Beuttenmuller & Dzaik, 2007), and although not shown in humans it has been linked to the onset of rickets when adsorbed in excess. Generally however, strontium is poorly adsorbed in normal human diets, although it may reflect the local strontium stratigraphy.

Strontium is best studied in calcified tissues which have a particular affinity for strontium where it is adsorbed in preference to calcium in the hydroxyapatite crystals. Some elemental studies suggest that the elements may help in the calcification of bones and the prevention of dental disease (Talwar & Srivastava, 2004).

Soil strontium levels higher than 350 ppm (mg/kg) are considered high, the typical concentration of soil in the UK varies between 2-2178 ppm, with a mean concentration of 75 ppm. There have been no known cases of adverse affects caused by strontium deficiency. Excess of strontium has not been shown to have adverse affects, although animal models suggest a link with the onset of rickets at very high levels. Strontium however is often supplemented in diets of people with poor bone structure, of which there have been no reported undesirable side affects (Marie et al., 2001).

Tin (^{124}Sn): Tin has no known role in the human metabolism, although it known to interact with riboflavin (Chatt & Katz, 1988). Tin does not appear to be toxic in itself, but the organotin compounds it forms can be very toxic, and have been utilized as antimicrobial and antifungal agents. In modern dental samples, absorbed tin levels may reflect dental treatment, stannous flouride, is an active ingredient in many toothpastes and mouthwashes Deficiency of tin has no known adverse effects in humans. Intoxication can occurs at levels of around 200 ppm (mg/kg) of ingested tin and leads to nausea, vomiting and diarrhoea. Reported intoxication is caused by the ingestion of foodstuffs contained within the acidic corroded tin plate food cans. There are no known natural causes of tin toxicity or prolonged effects (ibid).

Lead (^{208}Pb): Lead is a predominantly toxic element, intimately associated with industrial heavy metal pollution and pre-industrial metal working. Biologically, it exists naturally in minute trace amounts, and may have a biological function in some enzymes (Aras & Ataman, 2006). Contemporary levels are normally around 3 ppm, although notably lower in modern deciduous teeth possibly suggesting that lead exposure levels may be decreasing. Pre-industrial lead levels are very low, Neolithic samples, levels in teeth averaged around 0.3 ppm. In Roman, Anglo Saxon & Nordic samples lead levels of up to 10 ppm or more were occasionally found, although normally associated with occupational or acute exposure due to lead smelting, mining or metal working (Borsos et al., 2003). Although occasional anomalies, such as high levels in some medieval children, may also indicate pre-industrial environmental contamination (ibid). Deficiency of lead has no known adverse effects. Lead is a potent neurotoxin that accumulates in tissues, including calcified tissues, over time. Toxic concentrations of lead can cause neuron damage, blood and brain disorders. In teeth lead elevated levels are associated with enamel hypoplasia (Brook & Smith, 2006).

1.3.4 Elemental Isotopes of the Inorganic Faction of Hard Tissues

In addition to determining the health and diet of an individual, trace elemental concentrations have been shown to have an intimate relation to the local environment, particularly the geochemistry (Brown, et al., 2004; Curzon, 1975; Lakomaa, 1977), as particular geochemical variations or trace element profiles of an area can be established. The concentrations of the surrounding environment ultimately dictate the availability of these elements to the food chain and ultimately are reflected in the concentrations in man himself, whether in deficiency or excess.

Advances in analytical chemistry have opened up many new fields of exploration, and possibly one of the most exciting is the analysis of the geographic origins and migrations of human and other organisms with sequentially calcifying tissues (Cox et al., 1996; Evans et al., 1995; Lee et al., 1999; Lochner et al., 1999; Outridge, 1996; Outridge et al., 1995). This has only been made possible though the increasingly accurate and reproducible detection of very low levels of elements and their isotopes in these heavily calcified tissues coupled with an increased understanding of the deposition and retention dynamics of these trace elements in tissues (Aufderheide, 1989; Price et al., 1985).

The geology and geochemistry of the surrounding landscape also plays a very important role, with regards to both dietary uptake, but more distinctly in elucidating local geochemistry. The maturation of studies into chemical ecology show that the accumulation of strontium and oxygen isotopes in hard tissues reflects the landscape during the calcification and that each landscape has a unique elemental and isotopic signature. (Ambrose, 1990; 1991; 1993; Blum et al., 2000; Ericson, 1985; 1989; Price et al., 1994a; Schwarcz 1991; Schwarcz & Schoeninger, 1991; Sealy et al., 1991; 1995; White et al., 1998).

These studies merged with the developments in dental enamel research show these new techniques providing the first unambiguous and concrete methods for reconstructing human landscapes from early childhood and through the first decades (Cox et al., 2001; Cox, 1997; Grupe, 1998; Gulson et al., 1997; Sealy et al., 1995).

The ability to track individuals' natal home and then their ages at movement from their place of birth is based on the fortunate co-development and intertwining of three advancements: 1) Better understanding of the geology and chemical ecology of landscapes. 2) Better understanding of patterns of calcification of dental, enamel, dentin and cementum.

3) The development of chemical analytical methods that allow for the micro-sampling of enamel and other hard tissues.

The hard tissue samples provide a chemical signature of individuals at different ages at development. With samples taken from sequentially developing areas, one can track changes over the life of an individual. Cementum can provide information on annual changes (Evans et al., 1995; Hals & Selvig, 1977; Tsuboi et al., 2000) whereas primary enamel and dentin can provide a chronology of change from early years to late adolescence.

Analysis of the chemical composition of hard tissues may provide new and complimentary insights into 1) hard tissue chemistry and development, 2) diet and nutritional physiology, 3) the movement and migrations of individuals and 4) diverse environmental conditions such as lead poisoning. Paleonutrition for example has advance rapidly lately with the understanding of hard tissue development and chemical analysis, combined with an understanding of the ecological and physiological processes governing the deposition and retention of elements and calcified tissues (Aufderheide, 1989; Price et al., 1985).

The promise of this field is that isotopic and elemental concentrations in preserved hard tissues would reflect aspects of dietary intakes or nutritional status. Recent developments have demonstrated the potential of chemical studies of teeth. The relative delay of the maturation of the field compared to morphological and histological hard tissue analysis has been due to three main factors:

1) Initial preference was for bone as the hard tissue of choice for the studies. As results accrued processes governing elemental and isotropic incorporation and turnover in bone, not to mention post-mortem change, proved to be more complex than previously realised.

2) Until recently, methods were not available to detect the low levels of some elements and their isotopes accurately in heavily calcified tissue, not to mention the ability to chemically relate areas of enamel to known periods of development, in particular laser ablation that provides results related to the ring development of enamel and dentine (Outridge, 1996).

3) Bone element results were of limited value because of the lack of background information and controlled studies of ecological, physiological and biochemical processes. Enamel offers important advantages of highly regulates calcification geometry and inertness once formed, the understanding of its elemental compositions remains rudimentary.

1.3.5 The Archaeological value of Strontium Isotopes in Hard Tissues

As previously discussed, strontium is an element found trace amounts in calcified tissue, whose concentration probably reflects the availability of strontium in the local soil and ground water. What makes strontium important, however, is that it naturally occurs as different stable isotopes, the ratio of which varies according to the local geology. These ratios, passing unfractionated into calcified tissue at the time of formation, can be matched against the ratios of the surrounding geology, and depending on the calcified tissue used give us an indication of an individuals location over a certain period of time (required to tissue formation or turnover), and if they occur any possible migrations (Ezzo et al., 1997).

Clearly, as the geology dictates the ratio and availability of the strontium, it plays a pivotal role in determining an individual or populations residence patterns, and as such the local and intermediate geology will be covered in more depth, along with the nature and dynamics of strontium biogeochemistry.

There are sixteen radioactive isotopes of strontium though in nature strontium consists of only four stable isotopes. Strontium makes up approximately 0.025% of the earths crust, predominantly as Sr^{88}, at 83% of the total available strontium, the remainder made up of the 4 lesser isotopes Sr^{84} (0.6%), Sr^{86} (9.9%) and Sr^{87} (7.0%).From a geological perspective, strontium is a component of many rocks, though in highest concentrations in the rare minerals celesite ($SrSO_4$) and strontanite ($SrCO_3$) associated with hydrothermal deposits or pegmatites (Morgan et al., 2001).

As a group 2 periodic element, it is chemically similar to beryllium, magnesium, calcium and barium. In particular and intermediate between calcium and potassium, it may substitute for both not only in calcified tissues but as a lithophile in a variety of rock forming minerals including K-feldspar, potash-felspar, gypsum, plagioclase, calcite and dolomite along with some substitution with barium to form barite ($BaSO_4$) and witherite ($BaCO_3$). Muscovite, biotite, pollucite and hydrothermal microclines also contain high concentrations of strontium (Ahrens, 1948).

Strontium is relatively mobile, can move through soil, percolate with water, preferentially adheres to soil particles and the amount in sand is about 15 times higher than in interstitial water. Concentration ratios are typically higher (110 ppm) in clay soil. Naturally occurring rubidium consists of two isotopes Rb^{85} and Rb^{87} (27%) does not occur in its own minerals but is always found in potassium rich minerals and the caesium mineral pollucite as it can replace

potassium and caesium within the lattice structure, although it is limited in potassium minerals due to its size, and concentration can vary such as from mica to feldspar. Found in highest concentrations in lipidolite (0.5%- 3.0%) and amazonite (green microcline) (0.1-1.5%). Rubidium is also found in high concentrations in pollacite, muscovite, biotite and potash feldspar (Ahrens, 1948).

Sr^{87} is the end product of the radioactive decay of Rb^{87}, and that by comparison with the Sr^{88} concentration, a function of geological time, and can be therefore used to date geological structures. Due to the unique and variable nature of geographical distribution of rock types throughout the environment, coupled with the unique ability of strontium to pass practically unfractionated through the food chain and adsorbed into the calcified tissues. Hence, strontium isotopes, which show effectively no fractionation, represent the rocks from which groundwater came. The isotope ratios vary with the geology, from region to region, so it is possible to see if the values for the enamel (formed during the childhood and little altered) vary from one site, and if they match the values for the surrounding geology Similarly it is possible to compare the ratios for enamel with those of bone (which turns over and therefore represents in adults the water intake over the few years before they died) and gain an idea of population movements (Sealy et al., 1995; Price et al 1998; 2001).

1.3.6 The Biogenic Availability of Strontium and Human Uptake.

Enamel is believed not to change after the date of formation, which allows enamel to be considered as archive dependent upon the actual source material of the tooth. Chemical information based on metabolic action, preserved from a particular time frame which may allow a reconstruction of an individuals diet and even location at the time period. Since the relative mass difference between the different isotopic strontium's is so small, the fractionation is practically imperceptible as strontium moves through the food chain (Figure 1.34). Strontium isotopes in organisms, from plants to animals within a geological area are therefore a direct reflection of isotopic ratio of the surrounding environment (Beard & Johnson, 2000).

The additional benefit of the uptake into biological systems is that the rate of rubidium-strontium (Rb/Sr) decay is essentially stopped, as the radiogenic Rb^{87} is not taken up into the body at anywhere near the levels of strontium, and therefore has no further impact on the Sr^{87} accumulation, which is a particularly important factor in the study of the fossilized remains of prehistoric animals (ibid).

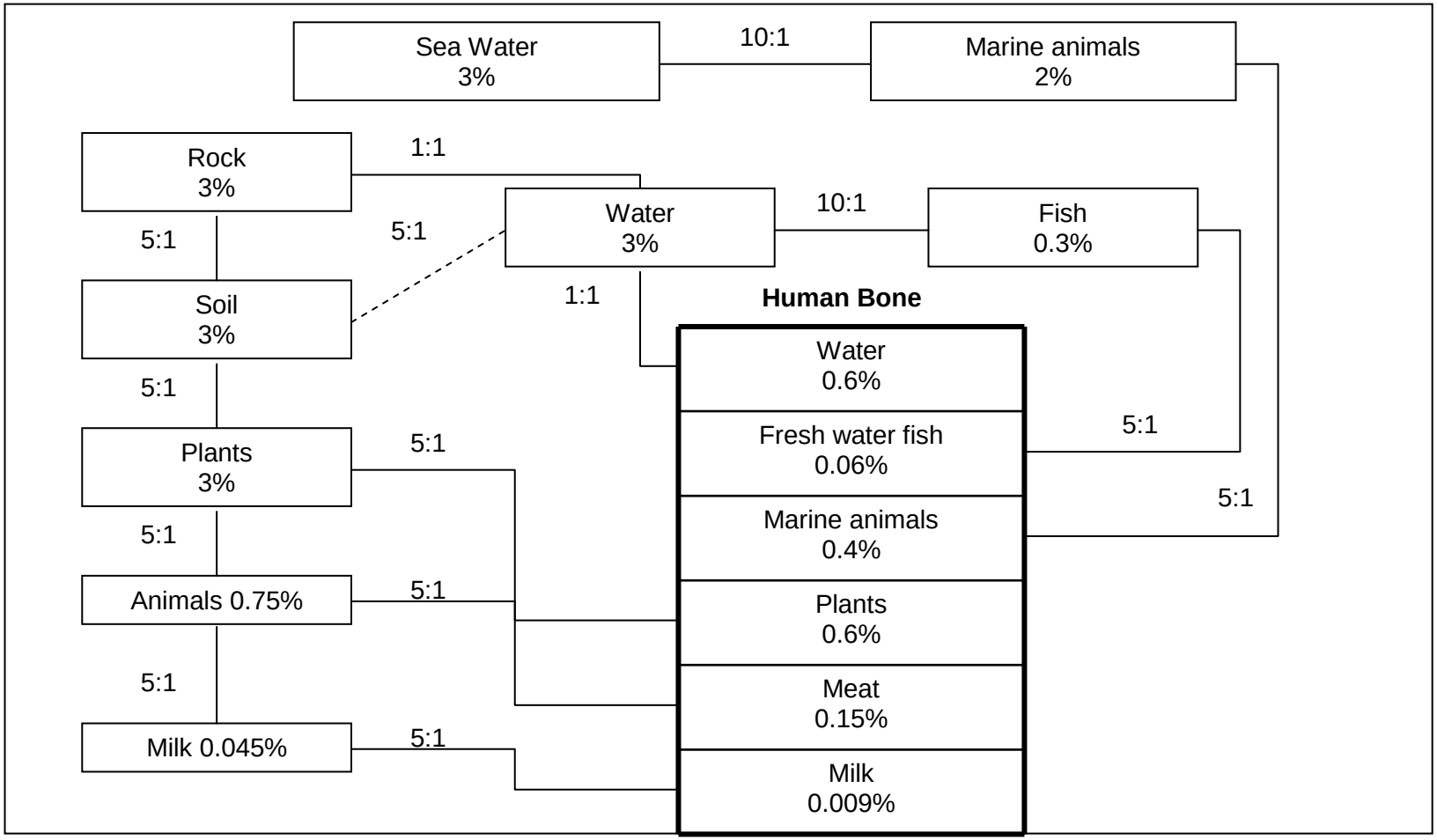


Figure 1.34: Fractionation of Sr/Ca occurring during Human Digestion and Composite Sr/Ca in Bone from various Diets. With knowledge of environmental levels of strontium and calcium and of fractionation dynamics, one can balance the relative input of strontium and calcium into bone or other calcified tissues. Ezzo (1994) suggests that strontium "is the only firmly established elemental model in bone chemistry analysis". Yet, this is not a simple system, nor has it been fully tested for either bone or dental tissues. Adapted from Price et al., 1985.

On average, 30-40% of ingested strontium is absorbed into the circulatory system, although at its highest in young children (60%) and in adults, particularly on low calcium diets, concentration decreases with age. As discussed previously strontium behaves similar to calcium, although not homeostatically controlled, but living organisms generally use and retain it less effectively. In adults 31% of the activity entering the blood (plasma) from the gastrointestinal tract is retained by bone surface; the remainder goes to soft tissues or excreted. Much of the activity initially deposited on bone surfaces is returned to plasma within a few days based on the updated biokinetic model that accounts for re-distribution in the body (Figure 1.34). About 8% of the ingested activity remains in the body after 30 days and this decreases to 4% after 1 year where most of the activity remains in the skeleton. The average 70 Kg individual contains an estimated 720 mg of strontium.

Although strontium ratios do pass on through the food chain, biogenic strontium ratios are generally somewhat lower than the surrounding isochron data, this has become known as the biogenically available strontium, and is currently one of the problems that arise in this research, which is the lack of discerning not only the surrounding strontium isotope ratios of the local bedrock and soil, but also determining the biogenic availability seen in the water, plants and small animals in the area in order to provide a “robust” measure of the local strontium ratio distribution (Price et al., 2002).

This information is particularly useful with regards to the Towyn-Y-Capel population, as the surrounding geological strata contain some of the oldest rocks in the North Atlantic, and consequently some of the highest strontium ratios in the area, which assist in differentiating the local population from those in the surrounding low strontium ratio areas.

1.4 Post Mortem Decay, Modification and Diagenesis

All the processes that affect tissues after death are collectively known as taphonomic processes. Taphonomy, originally coined by Efremov (1940), derives from the Greek *taphos* meaning burial, and *nomos* meaning law. This term, translated as "laws of burials", was introduced to paleontology to describe the study of the transition of remains, parts, or products of organisms, from the biosphere, to the lithosphere. Archaeologists study taphonomic processes in order to determine how plant, animal and human remains are differentially preserved. Understanding post mortem taphonomy is crucial to drawing scientific conclusions from fossilised remains.

During the post mortem period, tissues are exposed to attack by either environmental insults or modification by various agents, of biological, chemical and physical natures. This section examines the processes that affect biological tissues after death, and in particular those related to calcified tissues, and the impact these processes have on the structure, composition of hard tissues and the implications with regards to biomolecular preservation. Ancient DNA (aDNA) recovered from either historical or archaeological, remains usually differs from modern DNA samples in that it has undergone some form of degradation. Even in well preserved organic samples, degradation of DNA has occurs to some extent (Haynes et al., 2002; Mitchell et al., 2005).

1.4.1 Cell Death Decay, Autolysis and Soft Tissue Decay

The immediate decay of soft tissues is influenced by a variety of factors, such as soft tissue thickness, wounds and infections. Exomorphs tend to degrade more slowly than endomorphs, due to their larger mass to surface area. Wounds can facilitate the rate of degradation by increasing the available surface area for microbial degradation, and previously held microbial infections can provide an endogenous source of microbes, which may accelerate decomposition. In addition, the immediate external environment plays an important part, an exposed body for example, is prevalent to a rapid entomological colonisation. This significantly increases decomposition compared to a buried or protected body. If buried, another set of factors begins to dominate the rate of decomposition, in particular the soil biology and geochemical environment. Temperature is a major factor influencing the speed of biological and chemical processes (Jans et al., 2004).

The decay of soft tissue begins soon after death, with the autolytic breakdown of cells as a consequence of the failure of the cellular processes. The intracellular matrix is no longer maintained resulting in the breakdown of cellular components due to the unregulated activity of intra- and extra-cellular enzymes. . Most of the actual molecular damage to aDNA occurs during this period of degradation (Paabo, 1989). After autolysis, degradation continues through the action of microorganisms, fungi, insects, plants and animals. The rate of decomposition depends upon many factors, such as exposure to physical agents, temperature, pH, oxygen availability, the presence or absence of water and osmolarity. In short, the initial conditions of burial have the greatest affect on the long term preservation of protein and aDNA (ibid).

Following autolyis, putrefaction begins, leading to liquefaction of tissue (Di Maio & Di Maio, 1993). The process of liquefaction occurs through the breakdown of protein, carbohydrate and fat constituents. Putrefaction occurs quickly once the bacterial flora of the disrupted gut colonise the torso and spread through the vascular system. The external environment significantly affects the rate of putrefaction, particularly with regards to temperature, as breakdown is mediated by temperature dependent microbial enzymatic activity (ibid).

1.4.2 The Diagenesis of Hard tissues

Once soft tissues have been destroyed, and decomposition has reduced the corpse to the remaining hard tissues (bone, teeth, nails and hair), taphonomy takes on a totally different aspect, dependent on physical and chemical processes rather than biodegradation (Mitchell et al., 2005). This slow physicochemical degradation is particularly influenced by the chemical balance of the surrounding environment. This process is called diagenesis and was defined by Lyman (1994) as the alteration of biological tissue following interment and under the influence of intrinsic and extrinsic factors. Intrinsic factors affecting diagenesis are derived from the tissue itself and include the tissue type, size, porosity, chemistry and molecular structure. Extrinsic factors include soil pH, water (humidity), temperature and biological action due to bacterial, fungal and plants (Mitchell et al., 2005)..

These factors compromise the biomolecular integrity of the protein and mineral components of hard tissues. Figure 1.35 shows models of the molecular diagenesis of hard tissues, and the close association of chains in amino acid and DNA.

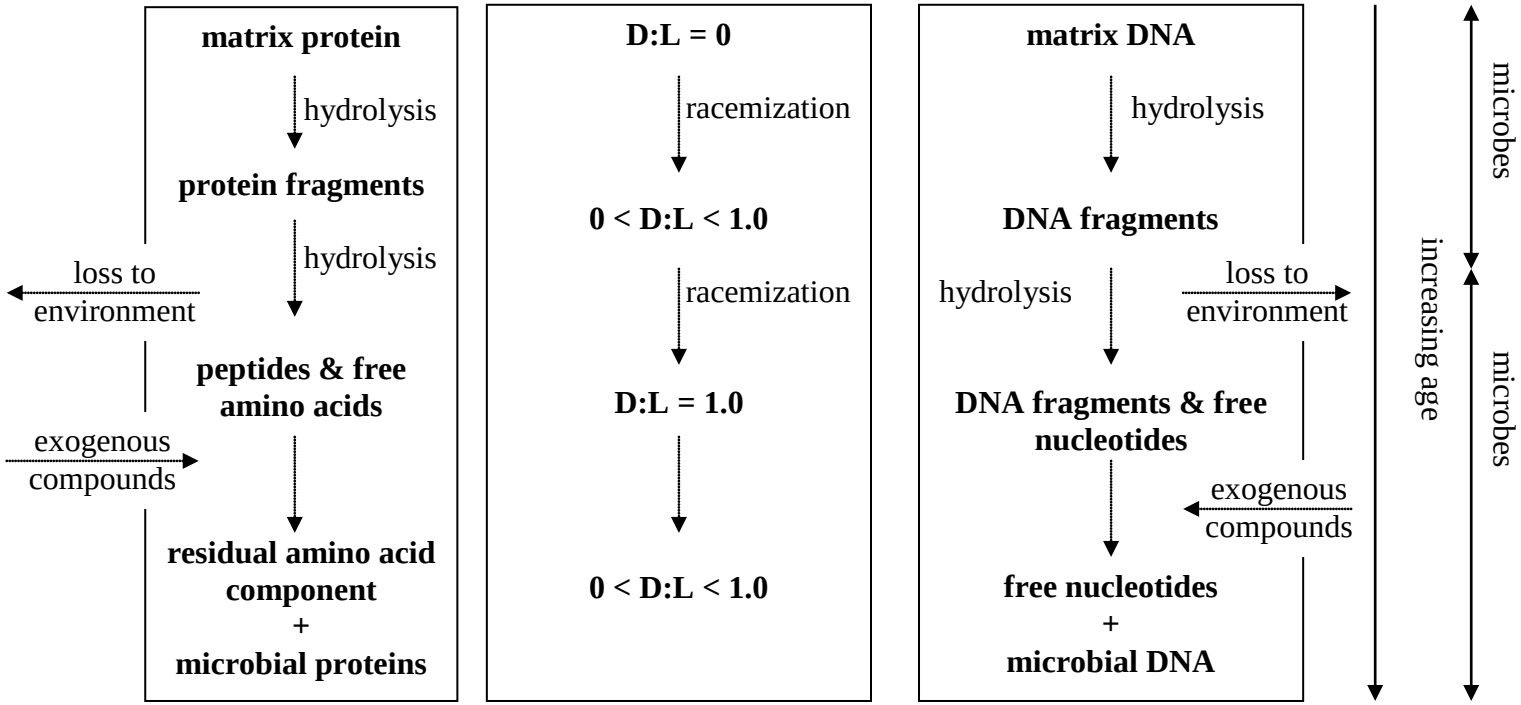


Figure 1.35: Flow Diagrams showing the Models of the Molecular Diagenesis of Hard Tissues.
This figure shows the interrelation between the environment, protein and DNA factions.The model on the left shows the cycle of protein content in hard tissues. The center block shows amino acid racemization resulting in conformation change from D to L enatomers over time. The D to L ratio corresponds over time to both the breakdown of protein (left model), and the breakdown of DNA (right model) over time. Model adapted from Rollo *et al.*, 2002.

The predominant diagenetic process known to occur in hard tissues after burial and a brief explanation of each are given below:

Compression:- This process involves the weight effect of overlying sediment layers. It can happen through a variety of contributing factors including seasonal affects and soil density.

Heating :- Especially combined with compression can cause defects such as cracks through rapid water loss and aid in the decomposition of organic matter

Re-crystalization:- Is the altering of the crystalline component of the hard tissue to a more crystalline form, that is stable in the local burial environment and therefore typically reflects surrounding soil conditions. The crystalline component may also suffer dissolution and replacement by a new mineral.

Infilling :- Involves the deposition of mineral inside the teeth but does not involve replacing the original crystalline components, instead filling the spaces left by the loss of organic matter.

Authigenesis:- Or the precipitation of mineral from dissolved sediment. Precipitation nucleates around the tissue resulting in a nodule or concretions (depending on size) of mineral encapsulating the tissue.

Organic Degradation:- Organic matter decomposition as a consequence of microbial, fungal or chemical attack.

1.4.3 Preservation of Hard Tissues

Teeth are generally more resistant to diagenetic modification, particularly in tooth enamel, but also in dentine, compared to bones. The resistance is therefore attributed to the inorganic faction. It has been observed that trace elements and rare earth metals may increase in concentration in skeletal remains over time (Lucas & Prevot, 1991). It is believed that the elements either replace the calcium ions, such as strontium, or more commonly absorb onto or trapped into the apatite crystals.

The higher concentration of apatite crystals, the robustness to diagenetic modification, the simultaneous presence of both of a dynamic and a non dynamic tissue types and the sequential nature of the tissue formation make dental tissue ideal for this study. For instance, it has been observed that the concentrations of the elements fluorine, zinc, silicon, lead, silver, copper and iron decreased from the tooth surface to the Dentine-Enamel Junction (DEJ), and that the trace elements fluorine, zinc, silicon, manganese, lead, silver, strontium, copper, iron and aluminium are higher in concentration in dentine than in enamel.

The long term affect of soil on calcified tissues, has been an area of great interest and speculation. Grupe & Peipenbrink (1989), determined that unless the soil is extremely enriched with one of the elements to be studied, that the microbial impact on the trace elements can be neglected This however does not exclude the potential for chemical modification, particularly in bone and dentine and possible contamination from organic or inorganic inclusions (Price et al., 1992).

Enamel Preservation

Under most conditions of archaeological burial, enamel structure is little changed. On occasion, enamel caps are all that remain of skeletal assemblages (Beeley & Lunt, 1980). Enamel is lost in very acidic soils, where, in contrast, the organic factions of dentine and bone survive (Stead et al., 1986).

As previously discussed (§1.3.3) tooth enamel is a very robust material, having a highly regulated calcification geometry, and a predominately inorganic and inert crystalline structure. Once formed enamel has been shown, in a number of forensic and archaeological investigations, to be is highly resistant to modification, both pre and post mortem, with particular reference to any diagenetic modification of the trace element composition (Kohn et al., 1999).

In general trace element concentrations are higher in enamel than in dentine or bone, which many have thought to be evidence of the predominately inorganic nature of trace element adsorption, as dentine and bone have a larger organic makeup. Human enamel hydroxyapatite crystals are estimated to contain more than 49 elements, most of which are barely detectable and appear to have no essential function in either the enamel formation or structure a number of theses are shown in tables 1.7 & 1.8. Elements which can exceed 100 μg per gram in bulk enamel include aluminium, barium, iron, lead, silicon, strontium and zinc (Curzon & Cutress, 1983).

Dentine Preservation

Fresh dentine is a very tough material, but archaeological dentine is frequently softened or brittle. This may result in tooth crowns fractioning or the flaking of the otherwise intact enamel (Beeley & Lunt, 1980). The poor structural integrity of ancient dentine appears to be related to collagen loss, occasionally showing signs of organic matrix staining (Falin, 1961; Poole & Tratman, 1978). In whole teeth and teeth sections viewed with the naked eye, reveal that affected dentine is chalky or opaque and found near the root surface or the pulp cavity.

Light microscopy of ground sections shows the damage to be associated with meandering canals throughout the dentine, about the diameter of the dentine tubules or larger caused by invading fungal mycelia.

Scanning Electron Microscopy (SEM) images of affected dentine and Back Scattering Electron (BSE) imaging demonstrates marked changes in mineralization (Bell, 1990). The dominant form of disruption is usually the scattered foci of diagenetic change, some with low densities, but many with higher densities than the intervening unaffected dentine, and usually following the incremental lines of intertubular dentine, peritubular dentine more often being left intact.

Preservation is difficult to predict. In some cases fully, fossilised teeth have better dentine preservation than much younger archaeological material (Doberenz & Wyckoff, 1967). It is even been hypothesised that diagenetic foci are repaired by chemical remineralization. At lower temperatures, the peritubular dentine splits away from the intertubular dentine. In archaeological material some trace elements, notably strontium, zinc and copper, have been found to have a higher concentration than those observed in modern samples (Lucas & Prevot, 1991).

Cementum Preservation

Cementum is often poorly preserved in archaeological material, often becoming detached from the dentine. Diagenetic change is similar to dentine and exposed at the root surface as a very thin layer, is greatly disrupted. Histology of cementum is very difficult, must be impregnated with resin before sectioning, using the simplest possible preparation techniques.

1.4.4 Molecular Degradation of Hard Tissues

Although the macromolecular processes of degradation are complex and vary highly between individual remains, the molecular processes involved in long term DNA decay are well characterised. DNA is inherently an unstable chemical compound, and decays spontaneously under certain conditions. Damage occurs at defined sites (Figure 1.36). The most common types of damage are due to oxidative and hydrolytic processes. These affect the structure and constitution of the DNA molecule in various ways, including depurination, base substitution, base loss, crosslinking and strand breakage. Ancient DNA possesses all these features to varying extents. These have a major influence on attempts to recover DNA sequences by PCR techniques (Mitchell et al., 2005).

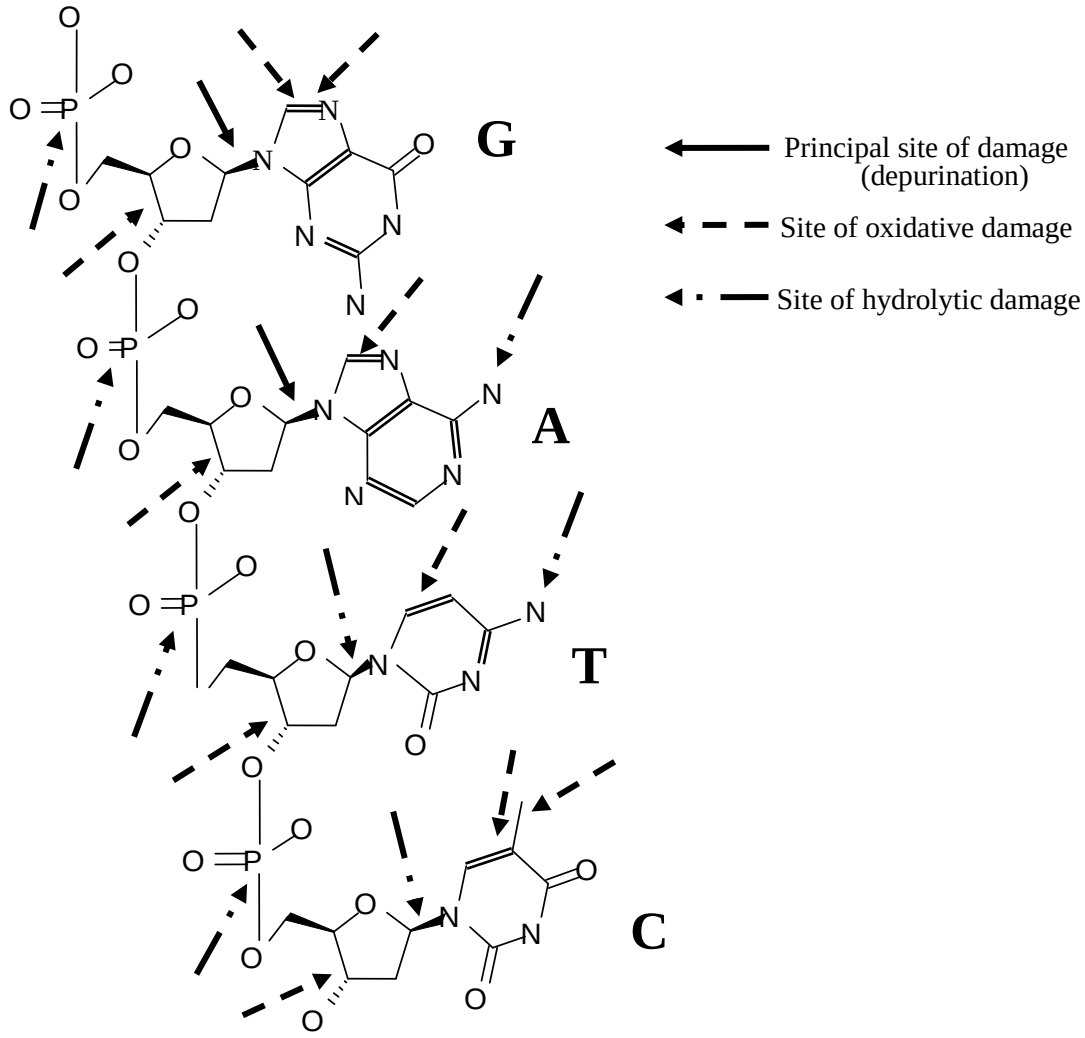


Figure 1.36: Sites of DNA Damage Shown to Affect Ancient DNA.
A short segment of one strand of the DNA double helix is shown with the four common bases. Principal sites of damage are indicated by solid arrows. Site susceptible to hydrolytic attack are indicated by dot-dash arrows and those prone to oxidative damage by dash-dash arrows. G, guanine; C, cytosine; T, thymine; A, adenine. Modified from Lindahl, 1993.

Oxidative lesions are induced by free radicals created by, amongst other things, background radiation. Major sites of oxidative attack are the pyrimidine and purine double bonds, which lead to ring fragmentation. The oxidized bases are believed to interfere with the elongation process of *Taq* DNA polymerase during PCR. For example, the oxidised pyrimidines 5-hydroxy-5-methylhydantoin and 5-hydroxyhydantoin are known to block *Taq* DNA polymerisation. Additionally, it is believed that deoxyribose residues are also susceptible to oxidation resulting in the fragmentation of the sugar ring (Lindhal, 1993).

Hydrolytic damage is characterised by the deamination of the DNA molecule, causing modifications of adenine, cytosine, 5-methylcytosine and guanine. The deamination products of cytosine (uracil), 5-methylcytosine (thymine) and adenine (hypoxantine) are of particular relevance as they lead to the misincorporation of bases during PCR amplification (A instead of G, C instead of T) leading and spurious aDNA results. Ultimately, hydrolytic activity induces hydrolytic lesions, strand breaks (Lindahl & Andersson, 1972) and the formation of DNA crosslinks (Poinar et al., 1998).

Apart from the fragmentation that occurs due to enzymatic processes during autolysis, nonenzymatic hydrolytic cleavage of the phosphodiester bonds in the phosphate-sugar backbone generates single stranded nicks. The nitrous base glycosidic bonds are subject to hydrolytic cleavage that results in abasic sites and nucleotide release. The site is thereafter prone to chemical rearrangement and strand breakage. As a result aDNA is rarely found in fragments larger than 500 bp in size and PCR amplified fragments rarely exceed 150 bp in size (Mitchell et al., 2005).

DNA crosslinks have been observed by electron microscopy and are produced by the formation of cross points of DNA (intra and intermolecular). Some complete structures that apparently consist of great quantities of DNA condensed and united by cross linkage have been identified by Paabo (1989). These structures could affect electrophoretic estimations of the DNA distribution, size (Paabo et al., 1990) and also block *Taq* DNA polymerisation. The main cause of DNA cross linkage is believed to be the Maillard reaction. Maillard products are formed by condensation reactions between sugars and primary amino groups present in proteins and nucleic acids. Treatment with N-phenacylthiazolium bromide (PTB), which breaks down Maillard product can allow DNA sequences to be amplified that could not otherwise be amplified at all due to glycation (Poinar et al., 1998).

1.4.5 Molecular Preservation in Calcified Tissues

aDNA integrity is not only characterised by molecular damage to the biomolecular structure of DNA molecule, but also by protective mechanisms that allow for the preservation of the DNA molecule. The preservation of biopolymers varies considerably according to their molecular structure, environment and diagenetic history (Eglinton & Logan, 1991).
As most of the molecular damage occurs during the period of autolysis, if this period is curtailed then the conservation of DNA is greatly increased. For example aDNA isolation has been most successful from mummified and frozen samples. However, in hard tissues the process of autolysis is not as pronounced, hence, DNA is better conserved in teeth and bones than in soft tissues.

Histological investigation of hard tissues has shown remarkable conservation of subcellular structures such as the cellular nucleus (Hagelberg et al., 1991; Cipollaro et al., 1998) and mitochondria (Hermann & Hummel, 1994). It may be that osteocytes and osteoblasts are involved in a rapid process of "cellular mummification" due to the limited water and active enzyme content of the cell and the high mineral content of the surrounding tissues. These cells are protected by this mineral matrix against physical, chemical and biological degradation (Hermann & Hummel, 1994).

It has also been proposed (Tuross, 1993) that DNA may be preserved by the presence of hydroxyapatite (an insoluble form of calcium phosphate). Nucleic acids have a high affinity for this compound (Sambrook et al., 1989), facilitated by interactions between the calcium residues and the phosphate groups from the polynucleotide skeleton. This interaction may provide protection for the DNA against enzymes present during autolysis. Dentine in the tooth has more hydroxyapatite than bone and this could explain why DNA survives well in teeth (Adler et al., 2011; DeGusta et al., 1994).

The survival of DNA in post-mortem tissues has been explored by Kelman & Moran (1996) who observed that fragments of nuclear DNA amplified by PCR are approximately 100 bp in length. Nuclear DNA is found within living cells in the form of chromatin, composed of basic units termed nucleosomes. Nucleosomes consist of chains of DNA approximately 160 bp in length and associated with histone proteins. It may be that the DNA surrounding the nucleosome is protected from enzymatic action, whilst the DNA linkers between each nucleosome are exposed to autolytic degradation. Thus, the DNA fragments extracted from ancient remains most likely reflect the length of the DNA protected by the nucleosomes (Alaeddini et al., 2010).

Protein Preservation in Hard Tissues

The proportion of carbon to nitrogen (C:N ratio) is one of the most important indicators of collagen (protein) integrity, and be indicative of the level of contamination. Ratios of C:N are calculated using isotope ratio mass spectrometry analysis, which identifies the elemental composition of a sample producing the weight percent of C and N. The C:N ratio of collagen in living tissues is based upon amino acid composition. It is 3.21 as there is 3.21 times more carbon in the collagen than nitrogen (Ambrose, 1993). The usual range of C:N ratios said to be acceptable for protein analysis are between 2.8 and 3.3 (Hedges, 2000). According to DeNiro (1985) and Ambrose (1990) however, ratios generally encountered in studies using bone range between 2.9 to 3.6. Some authors suggest greater flexibility in the placement of the upper limits for analytic purposes (Bösl et al. 2006), and suggest that whole bone nitrogen content is an effective indicator of the degree of preservation in calcified tissues (Coltrain et al. 2004). Although levels higher than 3.4 indicate probable contamination with carbon rich substances such as humic acid (Kennedy, 1988), C:N ratios of up to 3.9 are thought to be acceptable limit for collagen examination of archaeological material.

Potential contamination is the primary motivation for sampling compact bone where it is thickest and most dense (anterior femur) and also for the thorough purification of bone samples by removing humic acids and other contaminants. Diagenetic alteration of the naturally occurring balance can result in the misconstruing of data, which for example, can lead to the "mimicking" of trophic level effects resulting in the presumption of carnivorous diets when in actuality they are herbivorous (Grupe & Schweissing, 2001).

During diagenesis, the collagen faction of bone predominates until around 5% of the original protein component remains, at which point the non-collagenous protein (NCP) composition takes precedence. The NCP composition is typified by the complete loss of hydroxyproline and sometimes proline, a decrease in glycine to around 20% and large decreases in the amount of aspartic acid and glutamic acid (Stafford et al., 1991). The protein component of calcified tissues degrades to polypeptide fragments and ultimately to the free amino acids. Amino acids are more chemically stable than the parent polypeptides and may be recovered from skeletal remains (Briggs & Ellington, 1994). However, the effects of heat, pressure and dissolution influences the proportions of amino acids recovered from remains, and may hinder the determination of the original protein. Protein present may come from exogenous sources, such as the surrounding sediment, plant or microbial infestation. Despite this structural protein may be protected by the surrounding mineral faction, and, given the right diagenetic environment, can persist for several thousands years (Briggs & Eglinton, 1994).

Collagen structures, with amino acid compositions indicative of endogenous collagen have been found in teeth that date back as far the Pleiostone Eon (Wyckoff, 1972). Bone and teeth samples from the same Pliocene Eon skeletal assemblage have been compared and it was found that, although some bone samples showed the high glycine:alanine ratio indicative of collagen, most were composed of amino acid ratios similar to those in bacteria, and hence were due to infestation. However, in teeth from the same site, hydroxyproline, a characteristic collagenous amino acid not present in most proteins, was found alongside proportionally less proline and more leucine and glutamic acid, indicative of endogenous amino acids, that tends to support the theory that the tooth structure, despite its lower initial concentration of amino acids, tends to preserve the protein better than porous bone.

1.4.6 Amino Acids & Amino Acid Racemization in Fossilised Remains

As described earlier (§1.4) amino acids are the basic building blocks of all proteins, hence amino acids are principally found in such proteins in the soft and hard tissues in either living or degrading organisms (Lehniger, 1982; Voet & Voet, 1995). Amino acids in themselves can provide valuable information about the fossilised organism. Amino acids have been used to chronological date organisms before and after death (for determination of period of interment) for potential archaeological and forensic applications for over 30 years. Most notably, however, amino acids can be used in order to determine the biological condition of a particular sample and therefore its suitability for further investigation and use for techniques in determining important forensic and archaeological information from a sample, such as carbon dating and ancient DNA analysis. In addition to being capable of determining the biological condition of the calcified tissues, amino acid constitution can be used to determine the nature of the biological component, and in particular whether exogenous or endogenous contamination has occurred, which is instrumental in the accuracy and validity of techniques such as carbon 13 dating and ancient DNA analysis.

The structural modification of some amino acids, particularly Aspartic Acid, from left to right configurations has been shown to mimic the rate of DNA degradation and due to its predominance in the skeletal material, is easier to extract and assess and as such has been promoted as a screening tool for aDNA (Bada et al., 1984). Bada (ibid) was one of the first researchers to foresee the possibilities for the use of amino acid racemization as a screening technique for selection of samples for ancient DNA analysis.

As previously discussed, genetic material recovered from either ancient or forensic material is a useful and unique source of data. The sensitivity of the technique however, along with the inherent instability of DNA, has a large potential for misinformation and contamination that plague the field since its instigation in the early eighties. In addition, ancient DNA analysis can also be very difficult, time-consuming, and expensive. Comparatively, amino acid racemization is cheap, verifiable and consumes little material. Distinctly, however was the observed similarity between the racemization/ degradation of aspartic acid and the successful recovery of ancient DNA. Depurination, a hydrolytic reaction, is one of the primary causes of nucleic acid degradation and it was noted early that racemization is also influences by some of the same factors. It was determined at neutral pH, that the rates of amino acid racemization of aspartic acid and DNA depurination are almost identical at 45^{o}C to 120^{o}C (Poinar et al., 1996).

For aDNA analysis, the degree of racemization was determined for a variety of organisms and times in order to compare specific high rate amino acid racemization and the success of extraction and amplification of intact DNA (Poinar et al., 1996; Results of which are shown in Figure 1.37). It was discovered that the among the amino acids, it was the ratio of D/L enantiomers of the aspartic acid that had a continual link with aDNA recovery. D/L Aspartic acid ratios above 0.08, corresponded directly with the inability of recovering intact genetic information (Figure 1.37). It was here that Poinar established that a D/L Asp = 0.1 was the limit of successful recuperation of endogenous genetic sequences. They also indicated a relation between the ratio and the length of DNA successfully recovered. In the samples analysed with a D/L equal to 0.05, it was successfully shown to enable the amplification of sequences between 140 - 340 base pairs, presenting a linear relationship between degree of racemization and the degree of genetic decomposition (ibid).

Racemization can therefore be used, not only as an indicator of genetic and/or biological degradation of the sample, but also as an indicator of endogenous contamination (within the sample itself). Comparison of Aspartic acids with other amino acids such as alanine (Ala) or leucine (Leu), that have prolonged racemization rates, that can be shown as D/L Asp > D/L Ala > D/L Leu. If the ratios are outside of this relationship, then contamination may be present. An example of this was addressed by Poinar and colleagues (1996), with relation to proposed dinosaur sequences (Woodward et al., 1994) where it was found that the ratio of the D/L enantiomers of alanine were clearly higher than those of Aspartic acid, indicating the presence of exogenous amino acids (Figure 1.38).

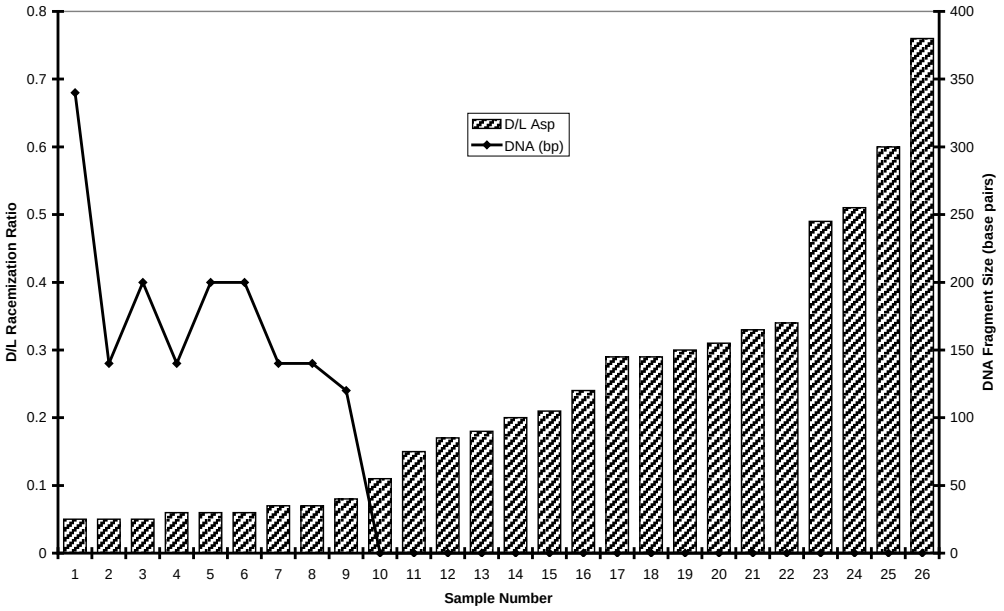


Figure 1.37: Aspartic Acid D/L Ratio against Published DNA Fragment Sizes. Note the rapid drop in successfully amplified fragment size as the D/L ratio of Aspartic Acid approaches 0.1 (~ sample 9-10). Figure produced from data from Poinar et al., 1996.

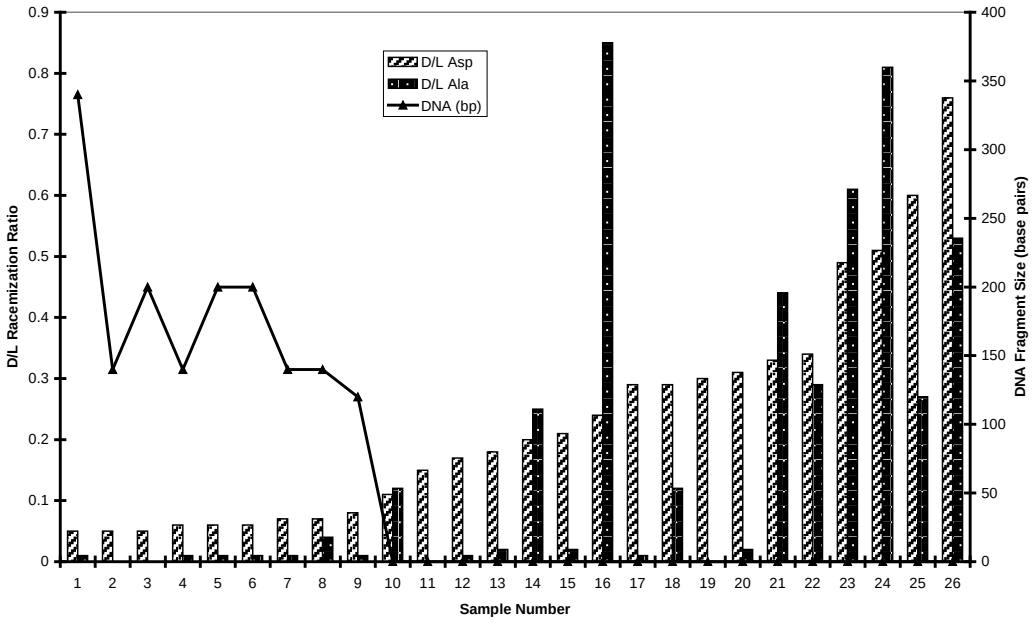


Figure 1.38: Aspartic acid/ Alanine D/L Ratio against Published DNA Fragment Sizes. Figure similar to Figure 1.36 however including the D/L racemization of alanine for comparison, where clearly no relationship with level and successful amplification of DNA exists apart from the necessity of low levels of D/L of alanine for successful DNA retrieval. Figure produced from data from Poinar et al., 1996.

1.4.7 Histological Analysis

Histology, the study of the microstructures of living tissues has a long history. Quekett (cited by Graf 1949) investigated histological components in bone and fossilised animal remains in 1849 and still others identified the characteristic bifringence, the difference between the highest and lowest of the two refractive indices can be quantified to give a "signature" bifringence measurement under the polarising microscope (Schaffer, 1889). One of the earliest studies of bone sections was obtained using a microtome after demineralising samples of mummified remains. In the studies by Graf (1949) it was noted that there was no discernable relationship between the age of the remains and microscopic preservation.

According to Paabo (1987), if a sample of tissue is to contain DNA, its histological state must be preserved. The first demonstration of the existence of DNA in human mummified tissue, was obtained using EtBr to show that the cellular nucleus was still present (Paabo, 1985). It is reasonable to suggest that a good cellular state of preservation is a prerequisite for the successful extraction of ancient DNA. However, DNA may remain protected through its association with histones, or histone-like binding proteins present in the cellular nucleus and mitochondria correspondingly, even if the membranes have been disrupted. The Windover brain tissue, for example, showed very few preserved cells, although they were still able to extract human DNA of around 10,000 bp with Alu (Paabo, 1986) and mtDNA (Doran et al., 1986) specific hybridization probes. It is therefore important to consider the histological analysis to be a good indicator of DNA preservation but where much more work needs to be undertaken to fully comprehend the association in soft tissues.

In relation to skeletal remains, the general appearance of bones, estimated a *grosso modo*, is not directly related to the preservation of DNA (Hagelberg et al., 1991; Faerman et al., 1995), although there appears to be a general relationship between the histological preservation of a sample and amplifiable DNA (Hagelberg et al., 1991) including the ability to amplify genes of only one copy (Cipollaro et al., 1998). This does overshadow the hypothesis that DNA is conserved better in bones by binding to hydroxyapatite, although a more detailed study into the association is lacking. For example, the relation between the histological preservation and endogenous DNA has not been studied in teeth, where the high hydroxyapatite content could be a factor responsible for the better quality of DNA (DeGusta et al., 1994).

The case against the cellular integrity as proof of DNA conservation, is the intact cellular nucleus and mitochondria in tissues is preserved in amber (Cano & Borucki, 1995) where

DNA recovery has not been realised, which puts into doubt the theory of cellular integrity as an indicator of DNA preservation (Colson et al., 1997).

It is hypothesised that the osteocyte is the most likely candidate as the source of aDNA as it is most numerous and deeply within the calcified matrix (Sampietro et al., 2006). The preferred samples for aDNA investigations are often taken from the inside of the compact cortical bone itself, although to date it is contentious as to whether or not this it being the source of the DNA or whether it is due to the reduced genetic contamination of exogenous material. Despite the conjecture regarding the primary source of biomolecules within the fossilised matrix, osteocytes lie within the bone matrix, where as osteoclasts and osteoblasts lie within internal surfaces (medullary cavity) (White & Folkens, 2005).

There are a number of studies that have used histological analysis of bone samples to assess the preservation of ancient bone (Colson et al., 1997; Cipollaro et al, 1998), and conversely have established that bones showing good histological preservation were more successful in the extraction and amplification of DNA.

1.4.8 Gas Chromatography-Mass Spectrometry (GC-MS)

Hoss and colleagues (1996) investigated at the presence of the 8 oxidative base modifications of the DNA extracted from bones and soft tissue of 11 specimens, covering a range of antiquity varying from 40 to 50 000 years. They found that the most common modifications were those derivative hydantoins of the pyrimidines and whose presence had a negative impact on the amplification of endogenous DNA. Although this analysis required a great deal of material, the negative correlation between the quantity of hydantoins and the efficiency of PCR is confirmed, the presence of hydantoin could serve as a useful indicator of the degradation of DNA. The number of samples, however, needs to be increased in order to establish the uniform nature of this observation (Hoss et al., 1996).

1.4.9 The Recovery of aDNA

The main characteristics of aDNA extraction can be summarized as follows:

1) Poor DNA Quality and Quantity

Due to organic and inorganic degradation, aDNA obtained from ancient tissues is highly fragmented and in very poor molecular condition when compared to that obtained from modern or fresh tissues. Furthermore, specific and non-specific hybridisation suggests that most of the DNA extracted is unusable.

2) Difficulty in Isolating DNA from the Surrounding Matrix

By virtue of the DNA preservation mechanisms, aDNA is often bound to a variety of organic and inorganic substances that have preserved the DNA from degradation for prolonged periods of time. These substances also complicate the DNA extraction process, where a delicate balance must be kept to preserve DNA integrity and to remove associated substances such as calcium and hydroxyapatite (Kalmar et al., 2000).

3) Inhibition from Surrounding Materials

The characteristics of some of the first aDNA extracts, combined with the differences in ultra-violet (UV) absorption (Paabo, 1985) and the brown colour of the extract (Paabo, 1987) gave the first indications that other substances followed the extracted DNA. Bone samples in particular (Brown & Brown, 1994) produced this brown extract despite the inclusion of further purification.

With the advent of PCR technology, it was soon observed that inhibition of the PCR amplification of aDNA was a common problem (Hagelberg, 1989). Interestingly, PCR inhibition in dental extracts seems to occur far less frequently than in bones (Zierdt et al., 1996). These inhibitory factors are considered as major hurdles in the field of aDNA research (Handt et al., 1994a) and can lead to false negatives, considerably increase the chance of external DNA contamination, through increasing the number of steps required for the extraction protocol and by elevating the number of PCR experiments required to investigate the sample. It is probable that inhibition is a multifactorial problem, depending on the sample and its own combinations of inhibitory substances. There are, however, some general characteristics of inhibition: a) it occurs in almost all of the samples found, b) it can be associated with either brown colour or blue fluorescence of the extract, c) it is not eliminated by organic extraction and d) it does not pass through the centrifugal filter that retains molecules over 30 000 Daltons.

The exact nature of inhibitory substances is unknown, although there has been conjecture. Paabo (1989) proposed that these were products of the Maillard reaction, started by condensation between proteins and reducing sugars particularly associated with soft tissues (Cooper, 1992) and coprolites (Poinar et al., 1998), which is not surprising since the Maillard reaction is common during the decomposition of foodstuffs (Dills, 1993). Other proposals include the presence of porophyns (Higuchi, 1992), tannines, humic acids or fulvic acids (Hagelberg & Clegg, 1991), and even the ancient DNA itself (by virtue of extensive damage).

4) Contamination from Exogenous DNA

In many cases of aDNA research, DNA is successfully extracted from remains, but most of the DNA extracted does not belong to the original organism but rather to microorganisims present during autolysis and from the surrounding environment (Hackett, 1981; Child et al., 1993). This DNA can not only contaminate genetic analysis, but also inhibit DNA analysis and amplification of DNA through competition or crosslinkage. A list of the criteria of aDNA authenticity, as agreed by leaders in aDNA research, has been formulated to assist in verifying results from aDNA prospecting (Table 1.9; see §1.5.3)

1.5 Ancient DNA and the Polymerase Chain Reaction

In 1983, Kary B. Mulis was working on inventing a method of sequencing DNA that was faster and more efficient, introducing certain modifications to the Sanger method (Sanger et al., 1977). The Sanger method was based on the incorporation of labelled nucleotides into one of the DNA strands through the hybridization of a primer or oligonucleotide to which a polymerase binds and begins to synthesize a complementary strand. Mullis's concept was a system that used two oligonucleotides flanking the sequencing zone and utilised both DNA strands.

This method was not a complete success for sequencing, but it was soon realized the greater potential of the concept, not to sequence DNA, but to amplify an unlimited copies of a certain DNA fragment. This method was thereafter known as the Polymerase Chain Reaction or PCR for short. This procedure became renowned for the simplicity, usefulness and that it was conceived 15 years after the consumables required for the implementation of the technique were available (Mullis, 1990).

Although mentioned in passing beforehand (Saki et al., 1985; Mullis & Faloona, 1987), it was not until 1988 (Saiki et al) that this method took the form that it has today, using a thermostable DNA polymerase, giving greater specificity, efficiency, sensitivity and reducing the time necessary for this procedure. The PCR method revolutionised genomics, and the impact of PCR on the scientific community was so great that it led eventually to the award of the Nobel Prize in Chemistry to Kary Mullis in 1993.

Soon after the initial discovery, the technique was applied to aDNA studies. Ancient DNA was already a point of interest just before the availability of the PCR technique, after pioneering in non-PCR based aDNA work in the early 1980's (Figure 1.39). Consequently, the field of ancient DNA research after PCR elucidation grew exponentially thereafter, both in terms of publicity and research. There are a number of general reviews and books published on the subject, some notable examples include Brown and Brown (1992; 1994), Paabo (1993), Richards *et al*., (1993; 1995), Koleman and Tuross (2000), Kaestle and Hornsburgh (2002), Cipollaro (2005) and Keyser-Tracqui (2005).

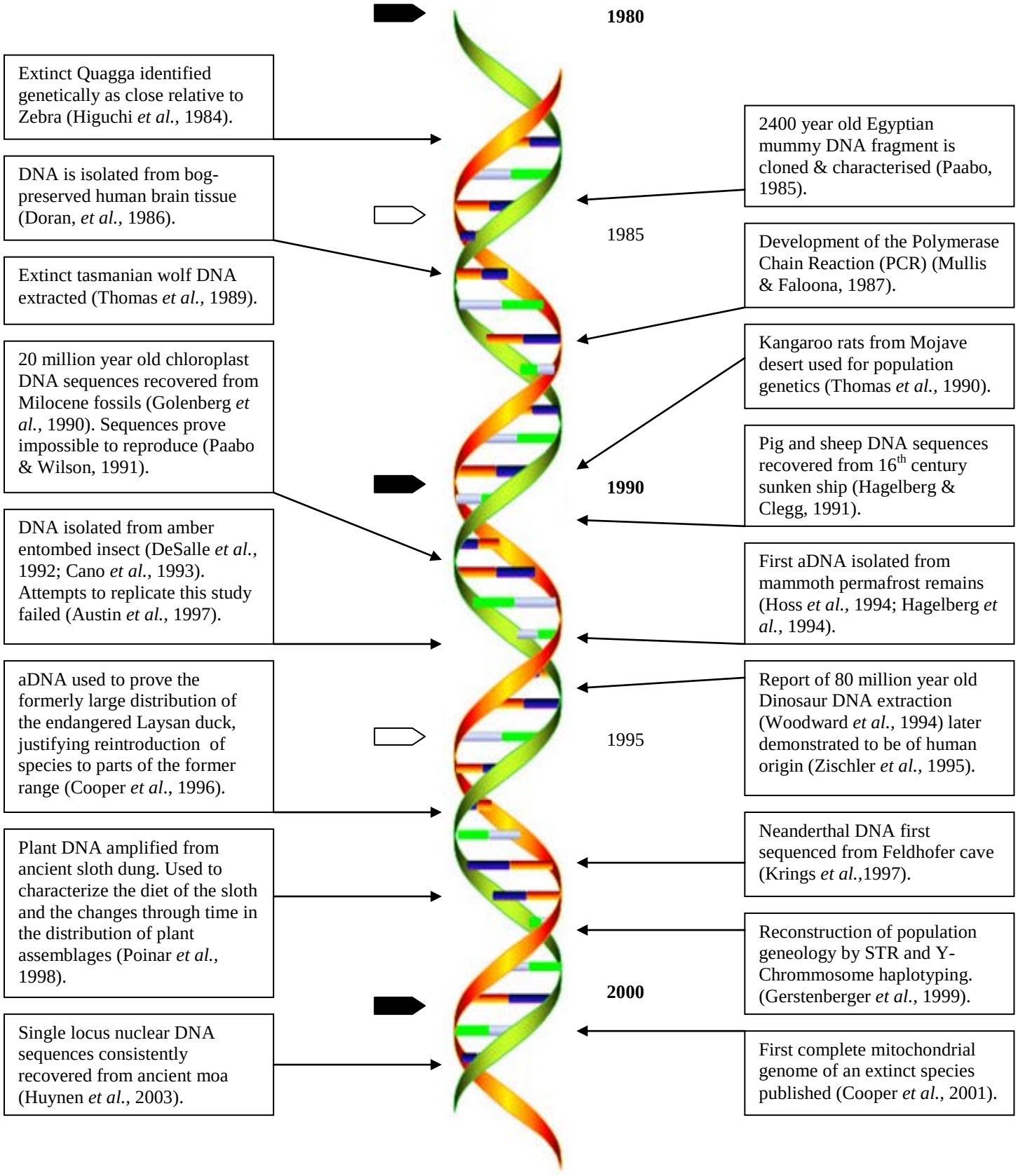


Figure 1.39: Chronology of Early aDNA Discovery
Significant developments in aDNA research over the last two and a half decades. Adapted from Shapiro & Cooper, 2003.

1.5.1 Practical Considerations in the Amplification of Ancient DNA

Soon after the first application of PCR to the study of ancient DNA, it was soon realised that the ancient DNA template provided some unusual challenges to the amplification itself, not in the least regarding the degraded nature of the DNA template (Mitchell et al., 2002).. In the following section some of these shortcomings are considered.

Sub-optimal Results

Despite the simplicity of the PCR method, sometimes results produced are not totally satisfactory, such as the low efficiency of amplification, amplification of different fragments thanks to incorrect hybridisation of primers that may also lead to other difficulties. Specific attention must be spent on using robust primers and an optimised and reproducible amplification process (Kaestle & Hornsburgh, 2002).

PCR Artefacts

Under certain conditions, the PCR can produce chimeric molecules or sequences that did not exist before. These molecules were created by the union of non-related segments. These types of fragments can confuse interpretation of results, such that some may end up to be the same size as the fragment expected. Its formation is induced by molecular damage (Paabo et al., 1990), hence this will lead to great problems when studying aDNA. (Lawlor et al., 1991; Francalacci et al., 1992).

Optimisation of PCR

Optimisation of PCR consists of finding the optimal condition that permits the greatest efficiency in obtaining the specific fragment of DNA with the smallest presences of artefacts, primer-dimers and non-specific amplification. This included the optimisation of the reaction mixture, temperature and duration of each phase within a cycle, the total number of cycles, and even the adequate design of the primers.

Components of the Reaction Mixture

Currently many commercially available DNA polymerases come as part of a kit offering optimised reaction mixtures for the chosen polymerase. These normally contain 10 mM Tris (pH between 8.4 and 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ gelatine and non-ionic detergents such as NP 40 or Tween 20 (at low concentrations such as 0.01%).

In some cases other constituents are added by the producer or even by the investigator to further improve the efficiency of the enzyme. The reaction mixture additionally contains the appropriate concentration of dNTP's (either present in the commercial mixture or added independently), the primers, the chosen DNA polymerase and the template DNA. The optimisation of the PCR is achieved through the empirical changes in the concentration of these components in the reaction mixture, normally 200 µM of dNTP's, 1uM of each primer and from 0.1 to 2 units of polymerase, in a volume of between 10 and 100 µL. The quantity of template depends on its origin, concentration and quality. For intact modern DNA 20 ηg to 10 µg can be used with good results (Mullis & Faloona, 1987). However normally around 500 ηg or less is used for amplification. Generally it is not recommended to add any of these components in excess, as it may reduce the PCR specificity.

A critical factor is the magnesium ion concentration, as an insufficient quantity can inactivate the polymerase, and an excess can reduce its fidelity (Eckert & Kunkel, 1990; 1991). The components of the reaction including the DNA template, proteins and chelating agents (such as EDTA) which can reduce the concentration of free magnesium, but can also be influenced by the composition of the primers and concentration of the dNTP's which tend to bind to the magnesium (Taylor, 1991). For these reasons, it is important to determine empirically the optimum concentration of $MgCl_2$ for each system, such that in many mixture kits, the magnesium is supplied separately as a 25 mM solution to be added separately to final concentrations of between 1 and 3 mM (ibid).

Low PCR Efficiency

Low efficiency is characterised by the production of less than sufficient or expected copies of DNA. The PCR being an exponential process is affected sequentially by negative factors in the first few cycles, leading to greatly reduced production. For example, at the beginning of the reaction there is far too many objective sequences, and that after denaturation, they compete against the primers and hybridize between themselves (Mullis & Faloona, 1987). Also, if the primers have not been adequately designed, and liable to partially bind to themselves, or do not hybridise properly to the target because the wrong annealing temperature. Additionally damaged target sites or blocking by secondary structures (Gibbs, 1990) can also reduce efficiency. In ancient DNA, the presence of inhibitors can reduce efficiency, although the expected amount of DNA produced is much lower than corresponding modern DNA, due to the low number of expected intact sequences.

Non-Specific PCR Amplifications

The amplification of segments of DNA that are not wanted are known as non-specific amplification, and the majority of which can be seen on an agarose gel as non specific bands of different molecular weights. Theses show as bands from larger to smaller than the fragment of interest. Such bonding often takes the form of an agarose smear and it is composed of a variety of DNA fragments of a range of different sizes. If the annealing temperature is incorrect, the primers can bind in a non-specific manner, binding to sites with far less than 100% homology. When these primers are extended, they produce a variety of reproducible, but non-specific products. Sometimes poorly designed Primer can give rise to an excessive occurrence of these products, despite the accurate selection of annealing temperature (Taylor, 1991).

Primer Oligomerization

This phenomenon more commonly known as primer-dimer formation consists of the polymerisation of the single stranded oligonucleotides to double stranded DNA. This process begins when the primers bind to each other's 3' end favouring the extension reaction (Chou et al., 1992) and normally occurs when there is either a non specific amplification reaction or in the presence of limited to know genetic template.

Amplification Artefact

Low copy number also directly affects amplification, because when the number of intact original sequences is very low, PCR may give positive results for ambiguous sequences and/or artefacts (Paabo et al., 1990) which could be equally related to contaminating sequences as with the phenomenon known as jumping PCR. In these cases, it is essential to clone the products in order to determine the true nature of each of the molecules amplified (or a selection thereof) (Handt et al., 1996). Some investigators have gauged the potential of this phenomenon as a means of gathering further information from aDNA by reconstructing aDNA sequences using jumping PCR.

PCR Inhibitors

PCR inhibition is a predominant factor in unsuccessful aDNA amplification, and this can arise from a variety of sources. Endogenous DNA inhibition by inhibitors such as humic, fulvic and even tannic substances that are isolated from the remains alongside the aDNA is widely reported.

In the literature, however, inhibition can arise due to carry over products from extraction procedures, including EDTA, SDS, Triton X-100, bile salts. Relief from PCR inhibition can be ascertained through stringent purification protocols after extraction, and from PCR additives such as BSA (Kneader, 1996).

PCR Contamination

A ubiquitous issue regarding the amplification of aDNA, due to the inherent low quantity of DNA extracted and the poor quality of the DNA template, and the extended PCR cycling required to amplify aDNA, make aDNA research particularly prone to contamination. Both pre and post laboratory acquisition of the samples, as even trace amounts of modern DNA are far more likely to be amplified than the degraded template present in archaeological material. Extended protocols of sterility and cleanliness have to be observed to reduce contamination from the researcher and the laboratory environment itself and are outlined in table 1.10.

PCR Carryover

In addition to low copy number, fragmentation and molecular damage to DNA, the control of carryover is also a problem, due principally to the small fragments sizes of the DNA analysed and the limited availability of the ancient DNA template compared to modern DNA template. Amplification of aDNA is an extremely delicate procedure requiring highly specific and sensitive primers and many cycles of amplification. Additionally, the resulting small amplicons (around 100 bp) have a higher chemical stability than larger DNA fragments and are thus less sensitive to DNA sterilisation and more likely to persist in the laboratory environment.

Further evidence suggests that intact modern DNA is amplified more regularly, than molecularly damaged ancient DNA, by PCR (Paabo et al., 1989) and even in the presence of an excess of aDNA, PCR favours the amplification of modern DNA. The reason for this is connected to the first critical thermo-cycles, where the aDNA is replicated with difficulty, mainly due to the crossover points and missing sections that lead to multiple replication termination events that prolong the amplification process by reducing the available polymerase activity.

Table 1.10: Established Criteria for aDNA Authenticity	
Physically isolated work area	To avoid contamination, it is essential that, prior to the amplification stage, all ancient-DNA research is carried out in a dedicated, isolated environment. A building in which large amounts of the target DNA are routinely amplified is obviously undesirable.
Control amplifications	Multiple extractions and PCR controls must be performed to detect sporadic or low copy-number contamination, although carrier effects do limit their efficacy. Positive controls should be avoided as they provide a contamination risk.
Appropriate molecular behavior	PCR amplification strength should be inversely related to product size. Reproducible mitochondrial DNA should be obtainable if single-copy nuclear or pathogen DNA is detected. Deviations from these expectations should be justified. Sequences should make phylogenetic sense.
Reproducibility	Results should be repeatable from the same, and different, DNA extract of a specimen. Different overlapping primer pairs should be used to increase the chance of detecting mitochondrial gene insertions in the nucleus (numts) or contamination by a PCR product.
Cloning	Direct PCR sequences must be verified by cloning amplification products to determine the ratio of endogenous to exogenous sequences, damage-induced errors, and to detect the presence of numts. Overlapping fragments are desirable to confirm that sequence variation is authentic and not the product of errors introduced when PCR amplification starts from a small number of damaged templates.
Independent replication	Intra-laboratory contamination can only be discounted when separate samples of a specimen are extracted and sequenced in independent laboratories. This is particularly important with human remains or novel, unexpected results.
Biochemical preservation	Indirect evidence for DNA survival in a specimen can be provided by assessing the total amount, composition, and relative extent of diagenetic change in amino acids and other residues.
Quantitation	The copy number of the DNA target should be assessed using competitive PCR. When the number of starting templates is low (<1000), it may be impossible to exclude the possibility of sporadic contamination, especially for human DNA studies.
Associated remains	In studies of human remains where contamination is especially problematic, evidence that similar DNA targets survive in associated faunal material is critical supporting evidence. Faunal remains also make good negative controls for human PCR amplification.

Table adapted from Cooper & Poinar (2000)

Molecular Misinformation

Molecular damage alone also plays a significant part in amplification strategy. Molecules with less damage, so called minor lesions, can promote errors in the copying process, which are further amplified. Such errors are not easily discernible, as the product is often much the same size as the target fragment, with some very important molecular flaws and misinformation. These errors can only be detected by molecular cloning and sequencing of individual PCR products (Paabo et al., 1989).

1.5.2 Crossover Contamination and Prevention

One of the greatest problems associated with PCR is exogenous DNA contamination. This may result in obtaining false positives, especially in samples with extremely low quantity of original DNA. As previously discussed, contaminating DNA can be introduced from a variety of sources, including other samples, but most commonly the investigator during any one of the pre-PCR steps (Capelli et al., 2003).

The problem is complicated significantly with PCR amplification, as each amplification reaction is capable of producing a great number of copies of the same fragment. This is a great potential for contamination, anything from the reaction constituents, as the subsequent extractions and amplifications (ibid).

These PCR products are called amplicons, and contamination with these is called carryover contamination, to differentiate from DNA contamination from a natural source (Kwok & Higuchi, 1989). The contamination potential is best evidenced in considering the power of the PCR. It is noted that each PCR reaction is capable of producing more than 10^{12} copies of the target DNA fragment in 100 µL. To put this into context, if this solution was uniformly diluted in the water of an Olympic swimming pool, each 100 µl of the swimming pool water would contain approximately 400 amplifiable DNA molecules. Furthermore, a tenth part of a microliter or the reaction mixture, such as that which would remain in a 100 µL pipette tip, could contain up to 10^{9} copies of the amplified sequence, to give comparison, a microgram of human DNA contains only 1.4 x 10^{5} copies of any one gene (Kwok & Higuchi, 1989). Even a picoliter (10^{-6} µL) could contain 10 000 molecules (Walder et al., 1993).

Therefore, contamination control has to be a very important part of research, to control this contamination a variety of protocols are put in place. It is particularly important to avoid the transfer of DNA between samples, and the controls, and extreme care with amplified samples.

The contamination by amplicons from one site to another can even be mediated by skin, or the clothes of the investigator (Kitchin et al., 1990; Rys & Persing, 1993). The conditions recommended for sample manipulation are similar to microbiological techniques for the management and cultivation of microorganisms (Kwok & Higuchi, 1989; Cimino et al., 1990b) or even more extreme (Kitchin et al., 1990) (Table 1.10). Additionally some precaution must be observed with the equipment for amplicon analysis, these include electrophoresis apparatus, transilluminator surfaces, centrifuges etc, as they may present some risk of contamination and can be cleaned when necessary with 1 M HCl (Kwok & Higuchi, 1989). Currently many authors also recommend other techniques, UV light sterilization, as an additional step to those mentioned, and surface bleaching and treatment with anti-DNA detergents specifically for surface contamination in PCR setup areas.

UV Light irradiation

One of the first techniques used to control contamination (Sarker & Sommer, 1990) is irradiation with UV light, primarily of the PCR constituents (non-DNA) and some equipment before PCR to inactivate the amplicons that may be present. UV irradiation (200-300 nm) induces a cyclobutyl ring formation between adjacent thymidines on the same strand forming a thymidine dimer. In the same way cytosine and cytosine–thymidine dimers are produced. These pyrimidine dimers distort locally the base DNA structure, leading to an inability of being a template for replication (Voet & Voet, 1992).

Assays on the effectiveness of UV was initially performed in 1990 (Sarkar & Sommer) on fragments of 700 bp, resulting in a very good results. However, smaller fragments (~250 bp) that the efficiency of DNA damage was reduced (Cimino et al., 1990). It was later demonstrated that the wavelength of the UV light, intensity and time of exposure are the important factors UV irradiation. An amount of 110 bp contamination was completely eliminated by exposing the solution to 5 to 10 minute at a distance of 2.5 cm from 254 nm light source (J cm^{-1}). Fox (1991) agreed with these results and concluded that with an adequate exposure, contamination can be reduced 1000 fold.

The size and structure of the DNA fragment, however, influences the ability of this and other sterilization techniques (Espy et al., 1993) and thereby, it is necessary to empirically evaluate the exposure time for each different type of amplicon (Fox et al., 1991). This is critical when it comes to UV exposure to PCR solution before amplification as prolonged exposure can damage the primers or other PCR constituents.

Espy (1993) recommends that fragments sizes of over 250 bp should be used to in this technique. In the case of aDNA research this would be unwise as the fragments investigated are normally quite small (less than 250 bp). Moreover, it may be inconvenient to attempt to use UV to sterilise all the constituents of the PCR reaction solution. Taq polymerase is very sensitive to UV light, as is the DNA template for obvious reasons, and mean that much care has to be taken when using this pre-PCR sterilisation technique.

These limitations have led to a number of observations by investigators that indicated that this technique is not a comprehensive method for the control of carryover contamination. It is beneficial rather to see UV as a component of a suite of techniques used for preventing for template DNA contamination rather than elimination (Kaestle Hornsburgh, 2002).

Cimino (1990) suggested that instead of screening before the PCR, that a selective destruction of amplicons after the technique would be favourable. This procedure came to be known as post-PCR sterilization. Using isopsorlen, that is activated by UV light and resists thermocycling,, and to introduce this into the PCR mixture pre-PCR. Once the thermocycling reaction is concluded, isopsorlen can be activated with UV light to produce extensive damage and prevent further replication. The damage sustained would not alter the analysis of the products by site hybridisation, or fragment size gel electrophoresis. However, this would effectively impede any sequencing of the fragment and possibly any further analysis based on restriction enzymes. Nevertheless, the substance does have a negative effect on PCR efficiency and to counteract this other substances may need to be added, that in turn may have an effect on the sterilization itself. These procedures using photosensitive substance may have a further role in the UV treatment of equipment and other materials, as UV irradiation is much less efficient on dry surfaces (Sarkar & Sommer, 1990).

dUTP and uracil N-glycosylase

One of the most elegant methods of selective elimination of amplicons consists of the implementation of the dUTPs in place of dTTPs for the synthesis of DNA during PCR, consequently leading to the incorporation of uracil in place of thymidine into all the amplicons. Thus, all the amplicons in the mixture can be eliminated from subsequent reactions by incubation with uracil N-glycosylase (UNG). The DNA containing uracil (uDNA) is degraded by the UNG, however, not effecting the normal DNA, such that primers and the sample DNA can resist the treatment without damage (Longo et al., 1990). With this method contamination can be reduced by as much as 3×10^9 (Rys & Persig, 1993), but it is not totally free from inconveniences.

Incubation with UNG occurs before thermocycling, but to inactivate the UNG enzyme it is necessary to extend the first denaturation step by 15 minutes at 94^{o}C, which may have a negative effect on the DNA polymerase (Glenn & Braun, 1992). The UNG may also possess certain post-PCR activity, requiring that the PCR tubes have to be kept at 72^{o}C after thermocycling, and rapidly placing at 4^{o}C (Rys & Persig, 1993). It is also not possible to use standard bacterial vessel cloning as they contain enzymes capable of degrading U-DNA (Glenn & Braun, 1992). Additionally, the procedure is expensive requiring the dUTP, UNG, which is more expensive than the polymerase.

A limitation of this technique associated with this investigation is the possibility of information loss associated with the use of this technique, specifically with unique or highly damaged DNA, this is although a question, however, that needs further investigation. One of the first observations is that aDNA in some cases has sites where uracil is produced by deamination of cytosine (Paabo, 1989), such evidence points to the fact that uDNA is in effect limited damage DNA, and in effect the use of the technique of incubation with UNG could lead do damage of the original damaged target DNA (or aDNA) as well as possible amplicon contamination, and hence the irreversible loss of important information, although this has not been proven significantly. The procedure also limits the effectiveness of the PCR depending on the length and sequence composition of the amplicon. Fragments larger than 150 bp and with a low GC content being more susceptible (Espy et al., 1993).

Ribose Residue on Extreme 3'end

This method also selectively eliminates amplicons. First described by Walder (1993), it consists of the utilization of primers with a modified 3' end bound Ribose residue, which produces fragments with an internal ribonucleic bridge sensitive to treatment with RNase or NaOH. Primers ending with any of the 4 ribose residues work in the same fashion as primers composed completely of DNA, and the polymerase is equally able to extend and copy efficiently. This modification does not influence the annealing temperature, and no protocols need be modified from normal PCR. This procedure allows for post-PCR DNA sterilization.

To sterilise pre-PCR, RNase A is used, however, it is necessary that the primers end in a cytosine (C) or uracil (U) and that the amplicons are denatured by elevating the temperature. RNase A is sufficiently stable at high temperatures, and could destroy new reaction products.

To avoid this, β-mercaptoethanol is included in the mixture before thermocycling to inactivate (irreversibly) RNase and thereby allowing the proper functioning of the Taq polymerase. RNase treatment can be used with the mixture with all the components, as this does not affect either the primers or the DNA template under investigation. With this treatment, contamination has been estimated to be reduced by 10^4 fold, and is only inconvenient in the need to add the β-mercaptoethanol prior to PCR to conclude the sterilization process.

Post-PCR sterilization consists of incubating the sample with NaOH, and can possibly reduce contamination by 10^4 to 10^9 times (Rys & Persig, 1993; Waldor et al., 1993), however, at this step the tubes are opened (to add the NaOH) still leaving a chance for the amplicons to be released during this step. Additionally the resulting fragments are shorter than with the normal primers, requiring internal primers for sequencing.

1.5.3 Ancient DNA PCR Strategies

To deal with the unique PCR hurdles that aDNA poses a variety of novel techniques have been used to study aDNA. These techniques include many, but specific consideration was given to Jumping PCR, real time PCR (RT-PCR) and multiplex PCR. Despite fragmentation and molecular damage that make ancient DNA difficult to work with, the control of carryover is also a problem, due principally to the small size fragments of the DNA analysed and the relative scarcity, leading to extremely sensitive PCR that can easily be contaminated.

These small amplicons are also more durable, less sensitive to DNA sterilisation and more likely to form aerosols and persist in the laboratory environment. Furthermore evidence suggests that in PCR, intact DNA is preferably amplified than molecularly damaged DNA (Paabo et al., 1989). It has been observed that even in the presence of a great deal of aDNA, that the PCR favours the amplification of the smaller concentration of modern aDNA. The reason for this is connected to the critical first cycles, where the aDNA is replicated with difficulty, with crossover points and missing sections that take more time to amplify and reduce the speed of the polymerase during these essential first cycles.

Additionally, molecules with less damage, or so called minor lesions, such as the deamination of bases, can promote errors in the copying process, which are further amplified. Such errors are not easily discernible, the product being often much the same size as the target fragment, with some very important molecular flaws and misinformation. These errors can only be detected by molecular cloning and sequencing of PCR products (Paabo et al., 1989).

When the number of intact original sequences is either very low or nil, PCR may give positive results for ambiguous sequences and or artefacts (Paabo et al., 1990) which could be equally to do with contaminating sequences as with the phenomenon known as Jumping PCR.

In these cases, it is indispensable to clone the products to determine the true nature of each of the molecules amplified (or a selection thereof) (Handt et al., 1996).

Some investigators have observed the potential for some of these phenomena, as a means to gather further information from aDNA, to reconstruct aDNA sequences using Jumping PCR and other strategies. Additionally some techniques allow for the quantification of the sample DNA, and to observe the accumulation of DNA and compare it in a specific ramping and cycle profile, to determine in a specific manner the relationship between endogenous intact molecules initially in the sample and at the end of the PCR. A brief description is given in the following headings.

On the other side, the DNA with less damage, will not be copied as well as intact molecular DNA, due to the fact that in the first few cycle of PCR (the critical cycles) the intact DNA will be copied and amplified faster, more efficiently and more often than the corresponding ancient DNA. Hence, after the first few cycles despite the higher original quantity of a DNA, most of the DNA present would possibly pertain to the modern sequence and therefore, in the following cycles most of the DNA to be amplified would be modern DNA.

Jumping PCR

When DNA molecules are either fragmented or damaged to such an extent as to block polymerization, as in the case of aDNA, there is a possibility of reconstructing the sequence through the recombinant union of different copied fragments, a process known as jumping PCR. This process starts when polymerisation is detained prematurely on sites that are damaged or ruptured, producing incomplete copies that may be later hybridised with other segments of the objective sequence. This process, over a number of amplification cycles generates a mosaic of sequences that range between the two primers (Paabo et al., 1989; Paabo et al., 1990; Handt et al., 1994b). The reconstruction of sequences through jumping PCR could be useful to amplify haploid genomes or mtDNA molecules that are homogeneous. However, when analysing molecules that could be contaminated, or diploid or polyploid genomes, the jumping PCR technique can generate erroneous sequences through in vitro recombination (Paabo et al., 1989; Handt et al., 1994b).

Furthermore, since Taq polymerase adds an adenine when it finishes the synthesis of a fragment, jumping PCR can also be used to determine the frequency, quantity and localisation of the genetic lesions in an ancient DNA sample. It detects sites where synthesis has been interrupted by observing the addition of illegitimate adenines and thymidines. If the quantity of damaged molecules is smaller than the quantity of intact molecules, these exchanges will not be detected through direct sequencing, but may be observed by comparing the sequence with others obtained by sequencing multiple clones.

However, if the quantity of damaged molecules is much greater than that of the intact molecules exchange can be seen by direct sequencing (Paabo et al., 1990). It thus also been proposed that jumping PCR could be used as a criterion of authenticity of ancient DNA as standard (molecular misbehaviour) (Handt et al., 1994b).

Real Time PCR

A further development of the PCR is the use of real-time PCR for aDNA quantification, where fluorescently labelled primers are monitored in real time for amplification of DNA. This allows a realistic estimation of the amounts of original and final DNA quantity, and detects any irregularities that may indicate contamination (such as an early exponential rise in DNA concentration indicative of contamination). This technique also allows for the incorporation of multiplex PCR allowing the combined detection of two or more specific targets and consequent estimation of quantity (Kefi et al., 2003)..

Although extremely effective, the use of specific fluorescent primers can be expensive and may affect further downstream applications such as sequence analysis. The use of SYBR real time detection systems allowed for the use of normal nucleotide only primers for real-time PCR. This significantly reduced the price of real-time PCR, and allows post-PCR analysis such as sequencing. This technique has been used to study aDNA, because of the ease in detecting contamination and because it gives a realistic estimate of DNA content when other techniques are not successful (Wurmb-Schwark et al., 2002; Kefi et al., 2003; Alonso et al., 2003; 2004).

Multiplex PCR

A relatively recent PCR technique, multiplex PCR enables the use of multiple primers to specifically target multiple loci in the same reaction. This technique has come to the forefront of genomic research due to significant advances in equipment and consumables available to the geneticist, specifically in improvements of equipment, particularly the thermocycler, with faster ramping times and smaller volumes, finally culminating in high throughput genomic research. The advantage when working with Low Copy Number (LCN) DNA is in the limited amount of extracted material required for the maximum amount of data. This technique has very recently come to the forefront of aDNA research (Gerstenberger et al., 2002; Alonso et al., 2003; Kefi et al., 2003; Wurmb-Schwark et al., 2004).

THE SPECIFIC GENETICS OF FOSSILISED REMAINS

1.6 Ancient DNA Targets in Skeletal Material

The selection of an appropriate target is imperative, particularly when dealing with degraded remains, where a number of factors reduce the quality and quantity of DNA to be amplified. According to some research and prevalent theories about DNA preservation in ancient material, particularly due to either initial autolysis or subsequent diagenetic breakdown, indicate that their may be areas of the genome that are more specifically susceptible to molecular damage and therefore prove to be inefficient or even unsuccessful targets where modern DNA samples would not be so afflicted. In addition, the stringent conditions of PCR reaction must be kept in order to optimise the targeted area, in many cases this can be only successfully done with very robust primers. This section will consider the targeted areas and further elaborate on the nature of these genomes, distinct advantages, reproducibility and previous research.

1.6.1 Use of Mitochondrial DNA as an Ancient DNA Target

Mitochondrial DNA offers some interesting characteristics that predispose it to ancient DNA studies. These include the complete characterization of its genome (Anderson et al., 1981), the high number of complete mtDNA copies in each individual cell, maternal line transmission (Giles, et al., 1980), a relatively high rate of evolution (Brown, et al., 1979; Parsons, et al., 1997), the presence of many polymorphisms in a relatively small area (Bonneuil, 1988; Cann, et al., 1984), absence of genetic recombination and even the ancestral insertion of mitochondrial DNA (mtDNA) fragments into the nuclear genome (Collura & Stewart, 1995).

Mitochondrial DNA has been extensively used in aDNA research of early humans (Bertranpetit et al., 1995; Comas et al., 1997) and other extinct organisms, which in turn also supports its use as a ancient DNA target due to the sizeable databases. The study of mitochondrial DNA has made appreciable contributions to the field of anthropological biology, and the studies of hominid evolution and migration (Cann, et al., 1987; Cavalli-Sforza & Minch, 1997).

Given the established pedigree in low copy number DNA and aDNA research, mitochondrial DNA analysis is the perfect tool for preliminary inter-population studies, particularly between different ethnic groups. Most aDNA studies concentrate on the highly variable mitochondrial control region otherwise called the D-loop, where three hypervariable regions exist, HV1, HV2 and HV3 (Handt, et al., 1998).

1.6.2 The Genomic Organization of mtDNA

The intimate association and long co-evolution of the mitochondrial genome alongside the host genome has led to significant differences in the major eukaryotic kingdoms. In animals, the mtDNA is normally a circular genome of around 16 000 bp encoding 37 or less genes. Mammalian mtDNA can be further characterised by protein rich structures within the mitochondria (Nucleoids), which may also be units of mtDNA inheritance (Garrido et al., 2003) and mitochondrial transcription factor A (TFAM) seems to be at least one of the proteins responsible for the packaging of mtDNA (Alam et al., 2003).

Human mtDNA is typically 16568 base pairs in length depending on the molecule age and haplogroup, where some insertions, deletions or certain highly variable tandem repeats may be present. mtDNA has two strands a guanine-rich heavy strand (H) and cytosine-rich light strand (L), and it codes for 13 proteins , 22tRNAs and 2 rRNAs (Figure 1.40). Most genes are transcribed from the H-strand but one subunit of complex I (MTND6) and eight tRNAs are transcribed on the L-strand. MtDNA has no introns, and with the exception of a few non-coding nucleotides between the genes, the only non coding regions are the displacement loop (D-loop) which encompasses the hypervariable regions 1 and 2 (HV1, HV2) and another-intra gene hypervariable region 3 (HV3). D-loop contains promoters for both H and L strands, which is used to produce polycistronic RNA transcripts which are cleaved to produce tRNAs, rRNAs and mRNAs. The transcription and translation of mtDNA are controlled by the nuclear genes (Falkenberg et al., 2002; Shoubridge, 2002; Casas et al., 2003; Rodeheffer, 2003). Nuclear transcription of the respiratory chain subunits may not be co-ordinated with the transcription of mtDNA-encoded subunits (Duborjal et al., 2002).

The genetic code of mtDNA is different from nuclear DNA, and there are several variations of the mtDNA genetic code (Barrell et al., 1979, Knight et al., 2001). In mammalian mtDNA, UGA codes for tryptophan instead of termination, AUA codes for methionine instead of isoleucine, AGA and AGG are terminators instead of coding for arginine and AUA or AUU is sometimes used as an initiation codon instead of AUG. These differences make the protein-coding genes of mtDNA unintelligible to the nucleocytosolic system, except for the MTND4L gene which does not contain any UGA, AGA or AGG codons.

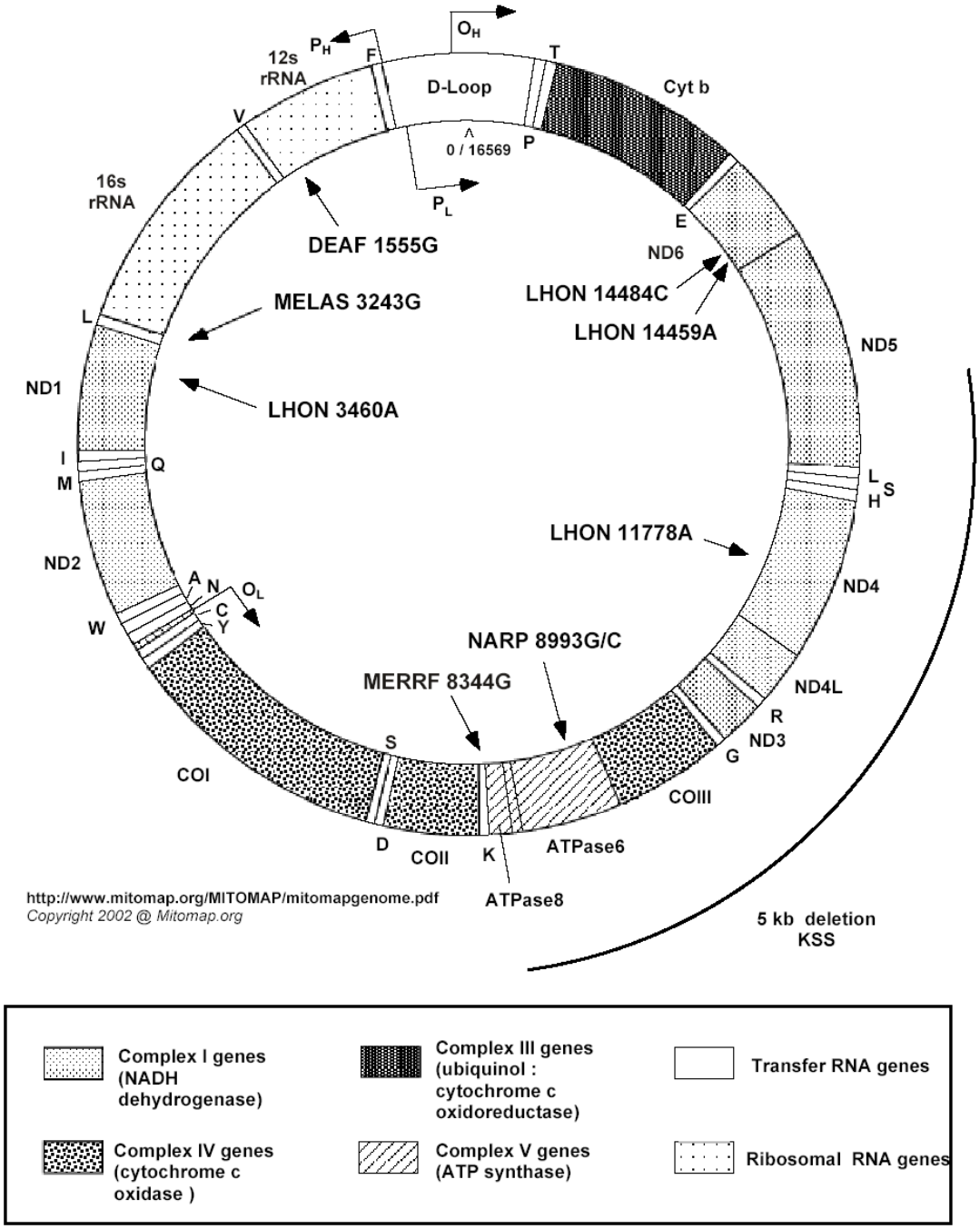


Figure 1.40: Schematic of the Structure of the Mitchondrial DNA Including Coding and Non-Coding Regions. Hypervariable regions 1 and 2 are distributed on either side (sequentially left and right) or the Point of Origin O_H and together make up the D-loop non-coding section of the mtDNA. Figure from Mitomap Human Mitochondrial Genome Database (ww.mitomap.org).

1.6.3 Elevated Number of Copies of Mitochondria DNA in Cells

According to Newman & colleagues (1996), observations of mtDNA *in situ* within a number of eukaryotes shows that mtDNA is highly organised within the organelle as grouped fibers within six differentiated centres called nucleoids. These nucleoides consist of DNA and binding proteins that have the ability to change the conformation of DNA strand. Each mitochondrion has a number of these structures, and within each cell, a number of these mitochondria, such that in a normal somatic cell there can be thousands of copies of mtDNA (Clayton, 1982; Shuster et al., 1988). This is significant in that the likelihood of recovering mtDNA from a tissue is higher than it is to obtain nuclear DNA, particularly in ancient remains (Paabo, 1989; Stoneking, 1993). In ancient DNA research in the selection of mitochondrial DNA because of the great quantity of the organelle DNA present in each cell is possibly the greatest reason to use mitochondrial DNA as the primary target. To give an indication of the number of mitochondria in hard tissue, the calculations in table 1.11 show the estimated mtDNA concentrations in 1 gram of modern bone.

1.6.4 The Maternal Line Transmission of Mitochondrial DNA

Mammalian mtDNA is predominately maternally inherited (Giles et al.., 1980). Just on the basis of probability, the numbers of mitochondrial DNA in the oocytes, with over 100 000 copies of mtDNA, whereas the sperm contains 100-1500 copies of mtDNA (Chen et al., 1995; Manfredi et al., 1997; Diez-Sanchez et al., 2003) tends to support maternal line transmission. In addition to this, although paternal mitochondria may enter the oocyte, they appear to be lost in the early stages of embryogenesis, soon after fertilisation, between the two and four-cell, possibly by ubiquitin-dependent proteolysis (Manfredi et al., 1997; Sutovsky et al., 2000). Studies show that the presence of paternal mtDNA could not be found in human neonates born even after in vitro fertilisation by intracytoplasmic sperm injection (ICSI) (Daanen et al., 1999; Marchington et al., 2002). It is possible, however, that the mechanism resposnsible may fail occasionally, leading to maternal/paternal mosaicism in an individual (Schwartz & Vissing, 2002) and in some rare cases at the blastocyst stage in some abnormal (polyploidy) human embryos generated by in vitro fertilisation techniques (St John et al., 2000).

Table 1.11: Estimation of mtDNA content in Compact Bone			
	Estimation of mtDNA content in compact bone	**Value**	**Comment**
1	Volume occupied by osteocyte lacunae	1.5% of bone	HgIP of human bone (NB slightly higher values are seen in bird bone)
2	Volume occupied by osteocyte lacunae	1.7–2.8%	
3	Size of individual osteocyte lacunae in µm	4.8–6.6 11.7–17.4 3–3.4	Width Length Height
4	Hence volume of osteocyte	200 μm^3	
5	Volume of osteocyte occupied by mitochondria	20%	In liver cells values of 15–25% reported
6	Skeletal Density of modern bone gcm^{-3}	1.4–1.7	Increases to 3.0 due to loss of collagen and water
7	Hence numbers of osteocytes per gram modern bone	5×10^7	Assuming a density of 1.47 gcm^{-3}
8	No of mitochondria per cell	1000	Ranges from 800-1500
9	Hence no of copies of mitochondria per gram	5×10^{10}	
10	No of copies of mtDNA per mitochondria	5	Literature values range from 2–20
11	Copies of mtDNA per gram of bone	2.5×10^{11}	Estimates vary from 3×10^{10}-1×10^{12}

[1]HgIP- Mercury intrusion porosimetry. Table adapted from Smith et al. (2003).

Table 1.12: Hypervariable Regions in the Human mtDNA Genome			
Hypervariable Region Designation	**Positions relevant to the rCRS**	**Number of variable sites***	**Hypervariable Region Length (rCRS)**
HV1 (D-loop)	16024-16365 bp	88	324 bp
HV2 (D-Loop)	73-340 bp	65	268 bp
HV3	438-574 bp	25	137 bp

*Data fromLutz et al., 2000

1.6.5 Heteroplasmy and Homoplasmy in Mitochondrial DNA

For the purposes of aDNA research, mtDNA can be considered as a single locus such that the alternative forms the alleles are constituted of a single sequence, and that the sequence varies in one or more nucleotide positions such that they may be considered distinct alleles of the same locus. The majority of individuals are homoplasmic, such that all the mitochondria in all the cells of the living organism have the same sequence or allele. To explain this phenomenon, it has been proposed that in mammals exists a mechanism that limits mtDNA divergence in all cells in such that all mtDNA sequences remain identical in between differentiated cells (Monnat & Loeb, 1985). This homoplasmia is surprising, their is diverse reasons why you should expect an accumulation of differences in the mtDNA molecules in an individual:

1) The number of mitochondria per cell could be up to 10,000, such that the loss of functionality of a small portion of of mtDNA molecules would not be a detriment to cellular propagation.

2) The genetic code of mtDNA is more degenerated than the nuclear genetic code, in that it is more tolerant to transcription and translation errors and can tolerate the persistence of mutations minimising the negative effect on the genetic code.

3) Apparently the γ-DNA polymerase has an exonuclease activity that exhibits an error rate of 1 base pair in every 7000 base pairs.

1.6.6 Lack of Recombination in Mitochondrial DNA

Recombination, a common and important feature of nuclear DNA that leads to new combinations of genes through crossing over between the loci during meiosis, occurs vary rarely if at all in mtDNA . Recombination accelerates the elimination of deleterious mutations and the incorporation of beneficial alleles during sexual reproduction, which is one of its main advantages (Butlin, 2002, Bachtrog, 2003). As previously discussed however, mtDNA in humans is predominately maternally inherited, and because of this clonal inheritance does not seem to have this benefit. Considerable investigation has gone into finding mtDNA recombination in humans and to date very little evidence (Merriweather & Kaestle, 1999; Morris & Lightowlers, 2000; Elson et al., 2001; Inman & Nordberg, 2002), despite such occurrences as nuclear insertions and rare heteroplasmy.

1.6.7 The Comparatively High Mutation Rate of Mitochondrial DNA

Mutations in mtDNA occur 10 times more frequently than in nuclear DNA, consequently mtDNA evolves rapidly (Brown et al., 1979; Wallace et al., 1987). Because of the proximity to the oxidative phosphorylation system of the mitochondrion, the mtDNA is exposed to far greater concentrations

of highly active molecules, and without the protection of the nucleus, the mtDNA is believed to suffer some oxidative damage by Reactive Oxygen Species (ROS). It has even been suggested that this may lead to gene-specific substitution rates (Nedbal & Flynn, 1998; Williams & Hurst, 2002). Notwithstanding, even more surprising is that it is assumed that mtDNA lacks effective DNA repair mechanisms, although some repair mechanisms do exists, it does not seem to be as effective as nuclear DNA repair activity (Croteau et al., 1999; Kang & Hamasaki, 2002; Mason et al., 2003). Furthermore, there has been some indication of a role of possible changes in repair activity and age (Chen et al., 2002; Stevenson et al., 2002). However, at least some mutations are caused context specific transient failures of the mtDNA repair system (Malyarchuk et al., 2002) and it is possible that any failure or mutation of the nuclear genome repair system replication may expose individuals to further mtDNA errors.

A point of interest arises from the mutation rate estimates, pedigree rate analysis for example gives a far higher mutation rate than the one estimated from phylogenetic studies (Parsons et al., 1997; Heyer et al., 2001; Howell et al., 2003) suggesting that a large proportion of mutations are observed in pedigrees are removed by selection before they can be observed as fixed polymorphisms in phylogenetic studies.

1.6.8 Mitochondrial DNA Insertions into the Nuclear Genome

A unique characteristic of the mitochondrial genome is that during its evolution within the eukaryotic cell, fragments of the mitochondrial DNA has integrated with the nuclear DNA, and have remained as pseudogenes in animals. Kuyl and colleagues. (1995) has observed these pseudogenes in a number of species from insects to mammals. Collura and Stewart (1995) determined through phylogenetic analysis that the transfer of a 3 kb fragment of cytochrome B occurred some 30 million years ago. Nomiyama and colleagues further estimated that 16S and 12S rRNA genes were integrated into the nuclear genome approximately 12 and 15 million years ago, respectively. The method that the mtDNA was transported into the nucleus is not clear, however, it has been observed that sometimes mitochondria are found within the nucleus of human cells, which may degrade liberating the mitochondrial genome, which may be integrated into the nuclear genome in manner similar to viral or plasmid DNA (Nomiyama et al., 1985). The main issue that arises with these pseudogenes is the effect they have of mtDNA phylogenetic studies (Smith et al., 1992) and as an insidious source of ancient DNA contamination (Collura & Stewart, 1995; van der Kuyl et al., 1995). The pseudogenes have also caused issues with the studies into mitochondrial mutation and disease, such as a link between Alzheimers disease and heteroplasmic mutation in cytochrome B, that in reality did not exist

1.6.9 Studies into the Variability of the Human Mitochondrial DNA

It is well known that the differentiation and establishment of evolutionary relationships can be established by comparison and, even more recently, these through the use of phylogenetics in order to finely resolve these biological relationships between species, through the additional use of DNA sequence and profile comparisons. The most characterised DNA target for phylogenetic work has been mtDNA for a number of reasons; notwithstanding, its high mutation rate, maternal inheritance and lack of recombination. It is also a highly conserved structure across large evolutionary trees and present in some similar form or another in all eukaryotic organisms. From the mtDNA conservation The evolutionary orders of mammals (Arnason et al., 2002) and the divergence times of humans and other primates (Glazko & Nei, 2003) can be estimated using mtDNA. Furthermore, mtDNA has been pivotal in determining intra-species divergence and evolution of different human populations (Richards et al., 2000; Cavalli-Sforza & Feldman, 2003). Further mtDNA studies coupled with the haploid paternal specific Y-Chromosome has provided combined information on the maternal and the paternal evolution of many populations (Lell & Wallace 2000; Ke et al., 2001; Helgason et al., 2003) including the British (Topf et al., 2006). Notable specific and large studies, including the genographic project, have been implemented using a combined system of looking at mtDNA, Y-chrommosome and autosomal analysis of sequence (Behar et al., 2007) in order to study human migration. Single Nucleotide Polymorphisms (SNPs) and microsattelites have generally confirmed the “out of Africa” hypothesis and that the human mtDNA lineages appear to originate from a single monophyletic phylogenetic tree which is rooted in Africa (Johnson et al., 1983; Ingman et al., 2000; Jorde et al., 2000; Caramelli et al., 2003). Due to the high mutation rate and the sequential accumulation of large numbers of nucleotide modifications (substitutions, insertions or deletions) in mtDNA lineages we can estimate chronological divergence on a time scale that matches the colonisation of different geographical regions of the world. Mutations are often seen as neutral, and to have evolved mainly by genetic drift, increasingly appear to indicate that specific selection pressures may have had a part to play in the human mtDNA story (Mishmar et al., 2003).

1.6.10 Mitochondrial DNA Haplogroups

The first studies into the human mtDNA lineages were based on Restriction Fragment Length Polymorphisms (RFLP) of the whole mitochondrial genome. These studies were able to screen 15-20% of the mtDNA sequence for variations (Chen, 1995). From this initial research the human mtDNA iwas differentiated nto different lineages, called haplogroups. The genetic differentiation based on RFLP remains the underlying classification of all the mtDNA lineages, although the resolution of the human phylogenetic tree has risen drastically with the advent of genetic amplification, advanced sequencing and profiling methods that can define haplogroups by even individual nucleotide differences.

Indeed, subsequent research has concentrated on the sequences of the hypervariable regions of the non-coding D-loop, HV 1, HV2 and the intra-coding region HV3 (Richards et al., 1996; Wilkinson-Herbots et al., 1996) (Figure 1.41) (Table 1.12). The haplogroup remains the core unit of the mtDNA phylogenetic tree, the principal clusters denoted with capital letters, with additional letters or numbers to denote lineages within the principal clusters (Torroni et al., 1996) (Figure 1.42).

1.6.11 Continent Specific mtDNA Lineages

The distribution of the major mtDNA haplogroups in different continents has facilitated the reconstruction of the human mtDNA dispersal in the "out of Africa" hypothesis (Figure 1.43). Haplogroup L, which is prominent in Africa, compromising ¾ of all African mtDNA, is hypothesised to be the oldest of the human mtDNA haplogroups (Wallace et al., 1999; Ingman et al., 2000) and closest to the recent common ancestor of all human populations (the "mitochondrial eve"), who presumably lived in central Africa 130,000- 200,000 BP. Haplogroup L is further subdivided into sublineages L0, L1, L2 and L3. Macro lineages M and N diverged from L, presumably in northeastern Africa or in the middle east approximately 65,000 BP, and the European haplogroups H, I, K, T, U, V, W and X (Torroni et al., 1996) subsequently evolved from N, whereas M and N contribute equally to the asian radiation of mtDNA into A, C, D, G, Z and Y. Subsequent dispersal of asian haplogroups, presumably by the Bering strait, lead to the peopling of the American continent by human haplogroups A, C and D (Derbeneva et al., 2002; Silva et al., 2002) whereas haplogroup B is believed to have arrived later via a coastal route (Starikovskaya et al., 1998; Mishmar et al., 2003). So it is believed that the majority of the haplogroups achieved their geographical distribution some 35,000-15,000 BP, excluding some demographic shifts that have occurred since then (Bandelt et al., 2001; Salas et al., 2002).

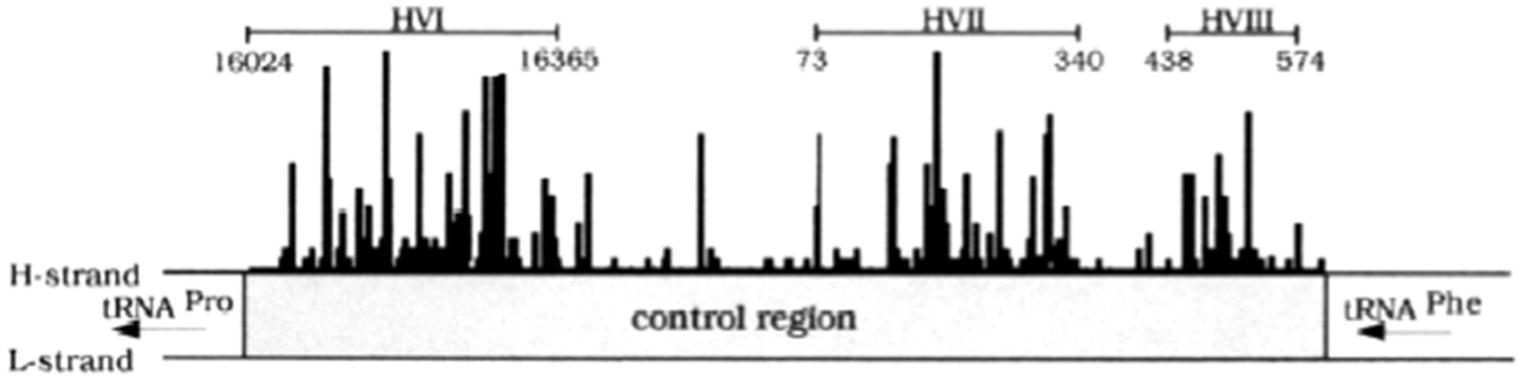


Figure 1.41: Graphical Representation of Polymorphic Regions within the Human Mitochondrial Control Region (D-Loop).

Figure shows the variation within the hypervariable regions HVI, HVII & HVIII for comparative purposes. Figure from Lutz et al., 2000

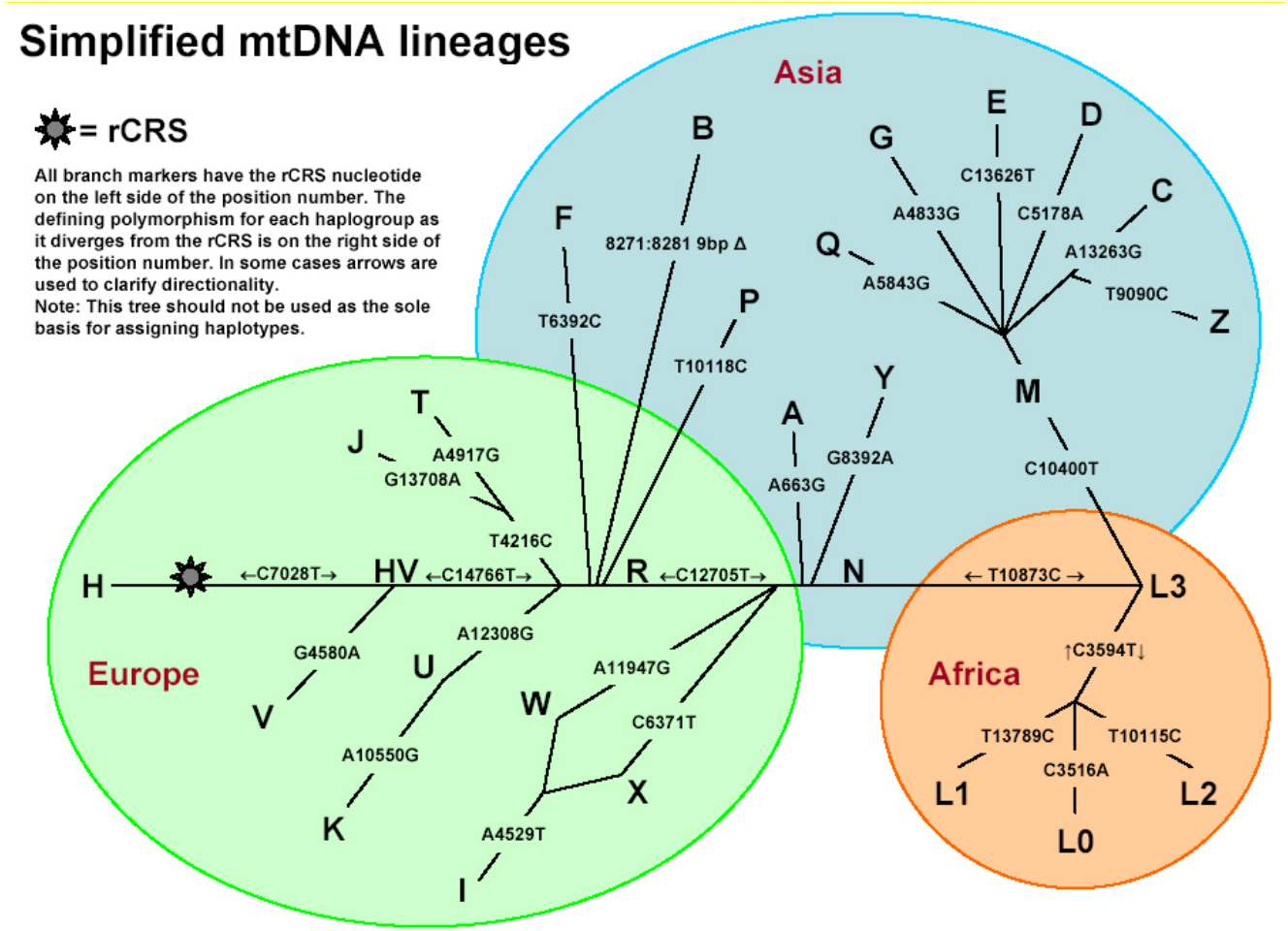


Figure 1.42: Simplified World mtDNA Lineage of Africa, Asia & Europe.
Figure from Mitomap Human Mitochondrial Genome Database (ww.mitomap.org)

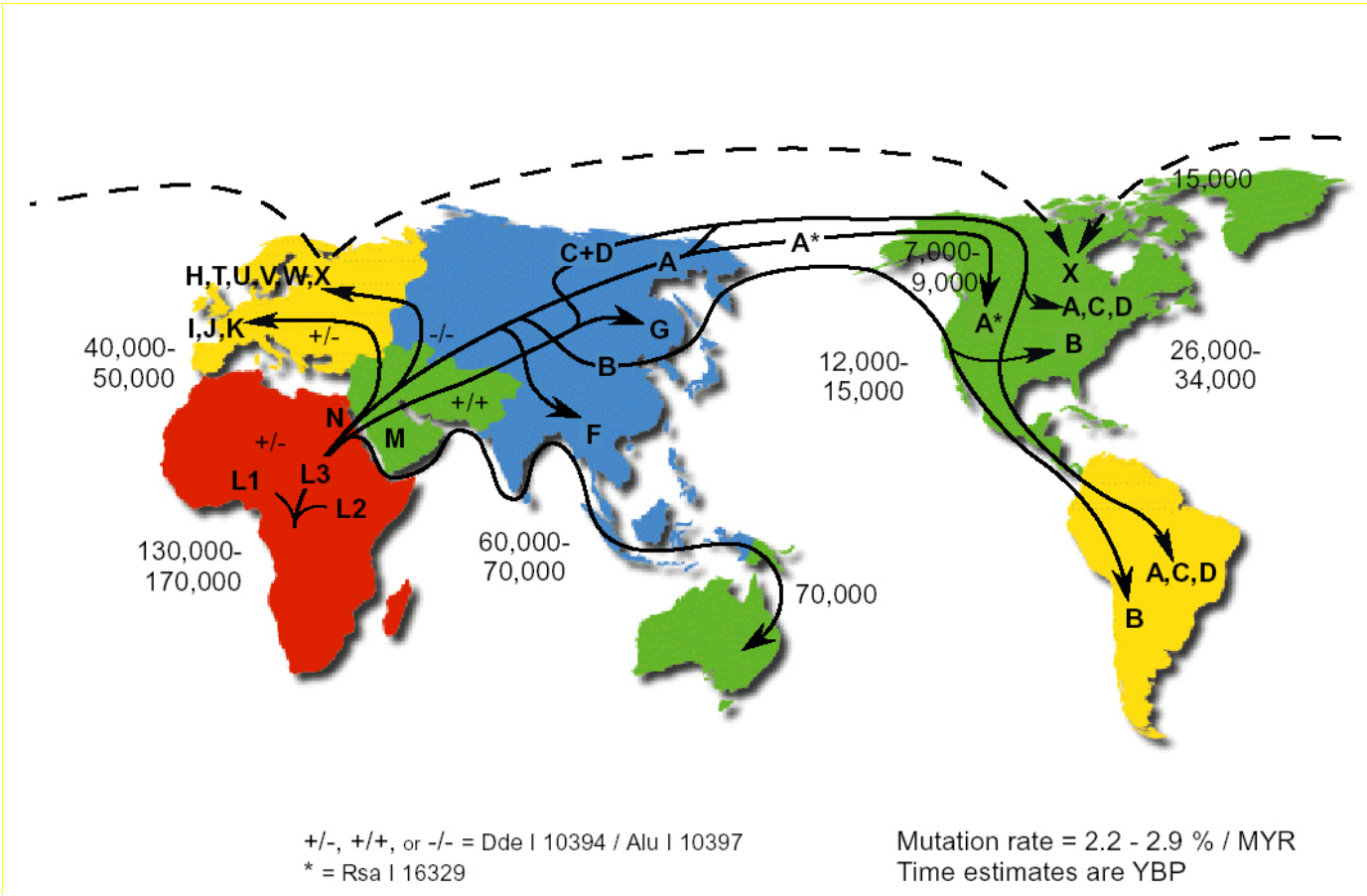


Figure 1.43: World Spread of Haplogroups and Approximate Chronology.
Figure from Mitomap Human Mitochondrial Genome Database (ww.mitomap.org)

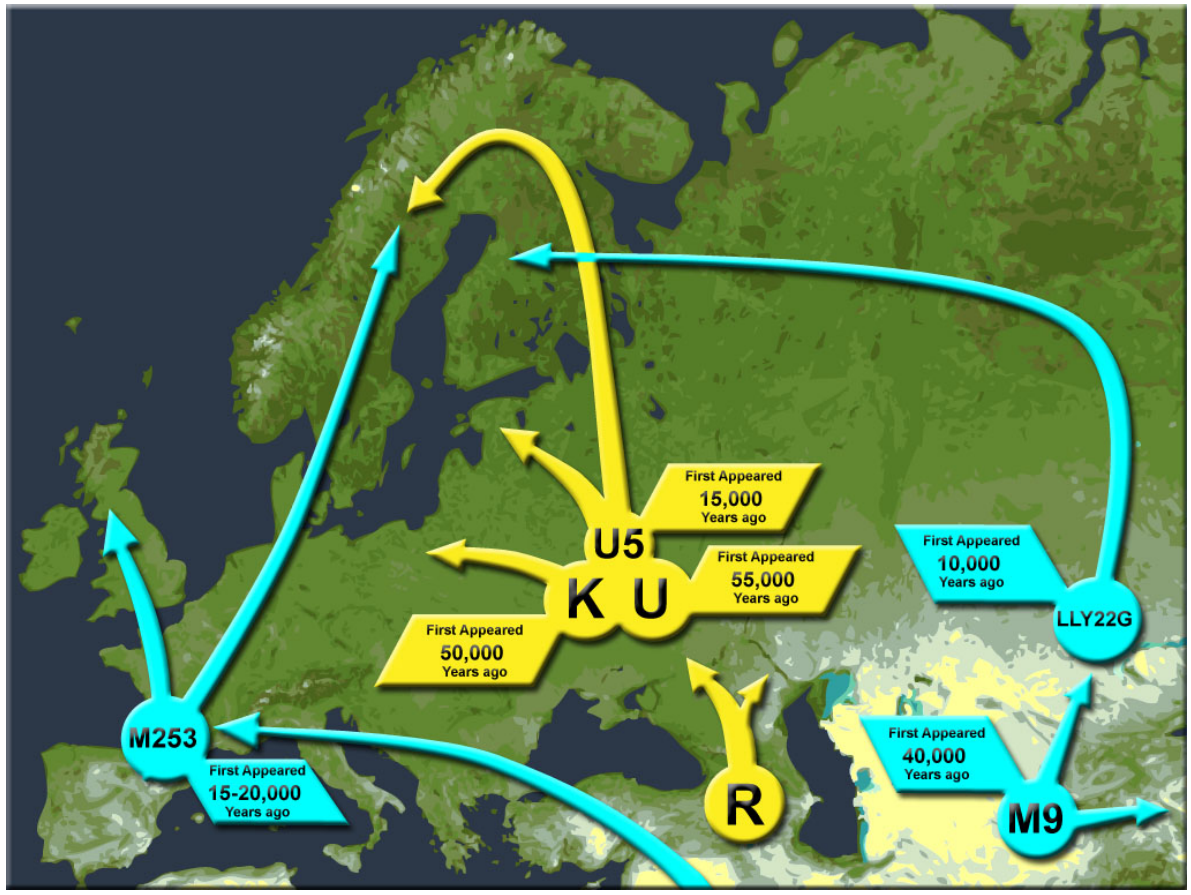


Figure 1.44: Pre Last Glacial Maximum Spread of Haplogroup K, U and U5 into Europe.
Figure is a graphical representation of the early mtDNA spread represented by (Yellow Arrows), specifically of the early U, K and U5 Haplogroups into Europe. Figure also shows supporting Y-Chromosome Spread (Blue Arrows). Based on National Geographics Genographic Atlas. Visualisation courtesy of Terker.

1.6.12 Chronology and Distribution of European Haplogroups

About 99% of the European mtDNAs belong to one of 9 mtDNA haplogroups, designated H, I, J, K, T, U, V, W and X (Torroni et al. 1996), these haplogroups are believed to belong to four mtDNA haplogroup superclusters, HV, UK, TJ and WIX (Richards et al. 1998). Six of the European Haplogroups (H, I, J, K, T & W) are essentially confined to European Popuulations (Torroni et al., 1994, 1996a) and probably arose after the ancestral caucasoids became separated from the modern Africans and Asians. Haplogroup U, although prevalent in Europe (mainly as U5, but also K) is also present in low frequencies in Japan, North African Berbers, Ethiopians and Senegalese (mainly as the sister group U6) (Ozwa 1995, Torroni et al 1996a, Passarino et al., 1998, Macaulay et al., 1999).

The European haplogroups phylogenetic tree is continually being more finely resolved with both modern and ancient genetic information, specifically the link between the haplogroup U and K, in which U has been expanded to include K as a subcluster (Figure 1.44). Haplogroup U is an anomaly in that it appears to have developed in situ and appears to be much older than the other haplogroups, with an estimated age of 51-67 kyr (Torroni et al., 1996a), compared to the relatively recent evolution of haplogroups H, J, T and V of 8 -30 kyr. (ibid). This tends to support an early African-Middle Eastern origin for haplogroup U and a more modern Middle Easter origin for the remainder. It is likely that apart from U and V that the remainder of the haplogroups were introduced by the proto colonization of 40-45 kya or by later arrivals in the middle/late Upper Paleolithic and Neolithic dispersals (Torroni et al., 1998; Richards et al., 2000). In addition, the major climatic changes would have affected the occupation of Europe, particularly in the Central and Northern areas (including the UK), where early paleolithic populations would either had to have retreated to the south or become extinct during the Last Glacial Maximum approximately ~20 Kya, followed by a gradual re-peopling of these areas ~15 kya. These observations are not only supported by archaeological dating (Housley et al., 1997; Richards, 2003) but by genographic work, particularly with regards to the U and V haplogroups (Torroni et al., 1998; 2001a).

In modern day European populations, haplogroup H is by far the most common, with a frequency of 40-50% throughout most European nations. Population frequencies of some modern countries along with some ancient populations are given in chapter VI (§ 6.4.3, Table 6.1, Figure 6.1) with specific regard to the Towyn-Y-Capel population.

Aim & Objectives

Working Hypothesis

Is it possible to analyze hard tissues (eg teeth and bones) from an ancient population, both biochemically and genetically, to ascertain a better understanding of this early medieval population from Ynys Mon, North Wales.

1.7.1. Overall Aim

The primary aim of this research project was to characterise human remains recovered from the archaeological excavation of the Towyn-y-Capel site in North Wales, in terms of their morphology and condition, and to then extract biologically significant data in order to gain archaeologically significant information relating the lives of these individuals. For example, it was hoped to be able to infer residence patterns and biological relationships within the Towyn-Y-Capel burial site skeletal population through the application of a range of scientific methods to a well characterised collection of bone and tooth samples. It was intended that a thorough, non-destructive characterization of samples, would enable the evaluation of different methods used in their analysis, including DNA extraction. To this end, samples were recorded and characterised in terms of their gross morphology and microscopic structure before analysis into the biological and chemical makeup was carried out.

1.7.2. Specific Objectives

The specific objectives of this research were:-

To record, document and otherwise maintain a comprehensive record of the fossilized remains before their use for destructive analysis in order not to lose important morphological and microscopic information

To conduct specific experiments to improve the lysis, extraction, purification and amplification of DNA from the fossilized remains with regard to the localized conditions found within the Towyn-Y-Capel population.

To extract, amplify and retrieve genetic information in the form of profiles or sequences from the skeletal collection at Towyn-Y-Capel.

To independently verify the results obtained, through the use of alternative methods including amino acid analysis in order to support the endogenous origin and genetic preservation of the skeletal elements analyzed.

To use the information ascertained to reconstruct biological links, origins and other archaeological relevant data such as the link between anthropology, elemental chemistry and isotopic analysis to unearth details regarding the individual and the population.

CHAPTER 2

SAMPLING AND MORPHOLOGICAL CHARACTERIZATION OF EXCAVATED REMAINS FROM TOWYN-Y-CAPEL

2 SAMPLING AND MORPHOLOGICAL CHARACTERIZATION OF EXCAVATED REMAINS FROM TOWYN-Y-CAPEL

2.1 Introduction

The Towyn-Y-Capel archaeological excavations provided a unique opportunity to sample an extensive skeletal collection of an early medieval population. Equally important to this study was the ability of the key investigator to participate in the recovery of skeletal materials from the site and to conduct on-site sampling for the biomolecular work along with keeping consecutive records from the site to the laboratory. This chapter describes the collection, recording and characterization of bone and tooth samples from the Towyn-Y-Capel site.

2.2 Site Methods and Materials

2.2.1 Skeletal Sampling

Sampling of skeletal material for subsequent DNA extraction was undertaken at site *in situ* as soon as the skeletal assemblage was encountered.

The selection of skeletal samples for analysis was based on the following criteria:

1) The sample should be quick and easy to collect, preferably at the first exposure of skeletal assemblage, to avoid or to minimise possible contamination by those excavating the site.

2) The sample should comprise a complete bone, for ease of sampling and storage.

3) The sample should not be of pivotal importance to the anthropologists in determining stature, sex, age, race and pathology.

4) Sampling would not interfere with the complete osteological investigations undertaken at a later stage.

On the basis of these criteria, it was decided to sample human patellae (knee caps), as these met all of the above conditions. Consideration was also given to sampling ribs where patellae were not available. Patellae were sampled *in situ* and removed asceptically with sterile tweezers and spatulas from the grave cut itself, before the skeleton was fully uncovered.

Sampling was conducted solely by the main investigator on all new skeletal assemblages as they were uncovered. A wore a face mask and two sets of gloves were worn. Samples were placed in pre-sterilised labelled brown paper bags that were inserted into ventilated polyurethane sample bags to prevent contamination and to allow the bone to dry naturally, as

storage of wet bone, particularly in plastic bags without ventilation, causes a humid micro-climate that can promote the growth of discolouring moulds.

Samples were stored at ambient temperature for the duration of the excavation in wrapped and protected boxes until their removal to the laboratory at the end of each archaeological session (normally 4-6 weeks).

The remaining skeletal components were carefully exposed, excavated and recorded *in situ* according to standard archaeological practice. Skeletal record forms were completed for all skeletal bones *in situ* in order to maintain effective records for later osteological investigations. All skeletal assemblages were retrieved from the site, and taken to the then Centre for Forensic Science (now the School of Forensic and Investigative Sciences) at the University of Central Lancashire for further osteological investigation.

It was soon determined from initial analysis in the laboratory (see §4) that the selected patellae were proving to be a very poor source for the extraction of genetic material, due to the trabecular nature of the material itself. However, cortical bones, in particular the dense femur, were of particular interest for the osteologists and were not easily considered available for destructive investigation. As a result, teeth were selected as an alternative source for untainted organic and inorganic information.

2.2.2 Non-associated or Loose Skeletal Finds

Samples not associated with specific human burials at the site (eg archaeological animal bones, loose human bones not associated with a burial), were also collected during the excavation. These were classified as non-associated or stray skeletal finds. Table 2.1 lists the collection of non-associated material recovered from the excavation. These samples were characterised by their small size and relatively poor physical condition. The preservation status of these materials varied from excellent to poor (Table 2.6). Most samples were either bone or tooth fragments of no more than 2-4 cm in diameter. This material was determined to have little anthropological value, and was therefore used for preliminary experiments and as site controls.

Table 2.1: Non-Burial Associated or Loose Skeletal Finds Recovered from Towyn-Y-Capel Site		
Find	**Context**	**Description**
126	349	Misc. long bone, hand, foot, skull, vert, pelvis frags. 3 molars
138	350	Unsided: tibia frag, sesamoid, MTT head, L: talus, calcaneus, navicular, cuboid, 2+3 cuneiforms, MTT1-5
138	350	R: talus, calcaneus, navicular, cuboid, 1+2 cuneiforms, MTT1-5, 4 prox ph's, 3 mid ph's, 3 dist ph's
155	374	3 unerupted molars, juvenile:3 long bone frags, 7 rib frags, 4 vert frags
155	374	Adult: 5 rib frags, 3 long bone frags, 4 sesamoids, 3 ph's (hand)
155	375	2 rib frags, 1 L temporal frag, MTT, 1 prox foot phalanx, L mandibular ramus frag
155	375	Juvenile L ulna
155	395	Unfused animal S1. small animal radius/ulna, MTT1, coracoid,
155	395	Juvenile L zygomatic and long bone diaphysis
155	396	1 skull frag, 4 long bone frags, 1 vert frag, 2 distal foot phalanges
159	351	8 femur frags, 1 juv rib frag, animal long bone
159	352	Juvenile verts and unidentified miscellaneous fragments
159	353	Rib frag, 3 human teeth, 3 animal teeth, 2 animal frags, 20 unidentified frags
159	354	Unidentified miscellaneous fragments
159	355	14 animal fragments, 21 juvenile fragments, 5 adult fragments, 2 deciduous unerupted teeth
159	356	Lump iron, 2 bags juvenile skull/teeth frags, juvenile humerus, 2xrib frags, 1 epiphysis
159	356	Adult:L+Rdistal humeri, distal radius, 7 phalanges, gtr multangular, MTC,
159	356	R mandibular ramus, small L patella, 5 tibia frags, 5 fibula frags, 4 animal frags
159	356	37 unidentified fragments
159	357	Distal fibula, carpals
159	358	Pubis, distal foot phalanx, neonatal ilium and temporal. 4 ear ossicles
159	359	2 animal frags, prox femur, dist phalanx, 1 skull frag, acromion frag, 15 unidentified frags
159	360	Distal R humerus, distal foot phalanx
159	361	Animal juvenile sphenoid, 6 unidentified fragments
188	219	Small finds top of back fill. MTC ends
188	376	2 unidentified frags
189	360	Stone and unidentified fragments
189	362	Humeral head, 2 rib fragments, cervical vertebra
189	363	1 unidentified fragment
189	364	5 animal fragments
189	365	3 unidentified fragments
189	367	2 unidentified frags
189	368	Unidentified miscellaneous fragments
189	369	3 animl frags, 1 large animal tooth
189	370	Unidentified miscellaneous fragments
189	371	Animal tooth, lateral incisor (root 1/4), 2 unidentified fragments
189	372	2 animal teeth
189	373	1 skull fragment
190	394	6 hand phalanges, 24 unidentified frags,
194	377	3 long bone fragments
205	159	Int. phalanx, L prox femur, temporal frag, diaphyseal tibia
207	399	Unidentified miscellaneous fragments
212	159	Poss frag derived skel S21 (521?): R prox tibia and small unident frags

Table 2.1: Non-Burial Associated or Loose Skeletal Finds Recovered from Towyn-Y-Capel Site		
Find	**Context**	**Description**
213	159	Foot and bone fragments' but actually R clavicle, rib, spinous process (vert) frags
216	159	Fragmented fibula, distal phalanx, carpal etc
218	159	Reburied bone fragments arm and pelvis' but actually L+R foot, R fibula and distal L fibula, all MTT and tarsals, 10 phals
222	397	Juvenile tibia. 1 adult prox foot phalanx
223	189	Small unidentified bone fragments
224	189	Animal bone
225	189	Bone - animal?
226	189	Bone' 2 frags - rib?
227	189	Tooth - animal?
228	189	Small unidentified bone frags
229	189	Small unidentified bone frags
230	189	Small unidentified bone frags
231	189	Unidentified bone frag
232	189	Small unidentified bone frags
235	189	Bone frag
235	226	Grave 185 Basal fill calcite? Tooth frag
236	189	Small unidentified bone frag
236	189	Small unidentified bone frag
238	379	Skull and thoracic vert frag
239	380	4 unidentified frag
239	381	3 unidentified frag
239	403	1 proximal phal (animal?)
241	189	Small unidentified bone frags
243	189	Small unidentified bone frags
243	189	Small unidentified bone frags
244	189	Small unidentified bone frags
244	189	Small unidentified bone frags
245	138	R distal tibia, R ilium frag (sciatic notch, auricular surface)
245	189	Animal tooth (labelled 'bone fragment')
246	252	Adjacent grave 335, sk107? Neonate femur, MTT/C, vert,2x unident. Adult: 2 cranial frag, 2 jaw frag, 7 rib frag, R scap frag, 4 vert frag, LMTC5, unident diaph frag
247	331	Adult fibula diaph, ribs, phal, juvenile skull frags, unident frags.
250	252	Adjacent sk109 grave 339. central mandible & maxilla
252	382	2 juvenile long bones, 1 dist phal, maxilla, 4 rib frags, 3 skull frags, 1 R frontal frag
252	383	1 first rib frag, 1 rib frag, 4 unidentified frags
252	384	18 unidentified fragments
252	385	Adult: clav, 2x mandibular frags, L lunate,MTC2, 2x skull frags, upper lateral incisor.
252	385	Juvenile: cervical vert, 2xrib frags, MTC. 7x unidentified frags
252	386	4 animal frags, 20 skull frags, 2 rib frags, 2 MTC's, 2 hand phals
252	386	2MTT's, 3 foot phals, Cuneiforms 1-3, juvenile: rib, MTC, Prox phal, Molar1
252	387	3 skull frags
252	388	5x juvenile vert frags, 2x juvenile skull frags, 1 animal tooth
252	389	2 unidentified frags
252	390	4 animal frags
252	391	Juvenile: 7 skull frags, 1 unfused cervical vert, 7 rib frags, broken L ulna, 1MTC, 1 pubis

Table 2.1: Non-Burial Associated or Loose Skeletal Finds Recovered from Towyn-Y-Capel Site		
Find	**Context**	**Description**
252	392	2 unidentified frags
252	393	Skull fragment
252	400	1 animal tooth, 2 animal frags, 25 unidentified frags
253	189	Small unidentified bone frags
254	189	Small unidentified bone frags
255	189	Small unidentified bone frags
265	402	Unidentified miscellaneous frags
295	404	Rib and skull frags
297	405	10 unidentified frags
298	406	Juvenile frags - temporal, long bone, vert.
315	409	Unidentified miscellaneous frags
315	412	3 animal teeth, 15 unidentified frags
327	411	3 unidentified frags
331	410	9 rib frags,4 skull frags,1 prox MTC, 1 distal 1st phal, L clavicle, temporal frag
332	413	4 iron lumps
332	414	4 animal frags, ear ossicles, 32 unidentified frags,
332	414	Juvenile: 9 rib frags, ulna, 9 phalanges, 2 skull frags, 11 vertebral frags
N/A	398	1 MTT, 10 unidentified frags
N/A	407	[E main section] humerus diaphysis, navicular, upper incisor, mid hand phal
N/A	408	White (calcined?) skull frags

Samples listed according to Archaeological Find number (Find) which geo located the samples recovery from a position within the archaeological excavation of Towyn-Y-Capel. Context refers to the soil matrix level of the archaeological site where the sample was found and relates directly to the site stratigraphy and sample age. Abbreviations used: frag- Fragments, R/L- Right/Left, vert-vertebrae, prox-proximal, int-intermediate, phal-phalanges.

2.2.3 Burial Population

During the excavations in 2002 and 2003, 122 complete or nearly complete skeletons were recovered from the site and classified as skeletal assemblages of archaeological importance and hence used for detailed anthropological investigations. A total of 103 burials were from simple Dug graves. These were dug through a humic soil layer into sand. In the lower stratigraphy 19 skeletons were recovered from several cist graves. Table 2.2 lists all the skeletons recovered from the Towyn-Y-Capel excavation, including the morphological analysis of the skeletal population from Towyn-Y-Capel. Table 2.7 lists those patellae and teeth samples collected at the site for further use in this investigation. Osteological analysis was performed by Anthropologist in charge of Towyn-Y-Capel Michael Wysoki and colleagues at University of Central Lancashire.

The state of the remains was defined along two distinct parameters: Preservation & Condition. Preservation refers to the approximate percentage of the total human skeleton that was present and is defined as: Poor if 0-39% of the skeleton was present, Moderate if 40-69% was present and Good if 70-100% was present. Conservation refers to the gross morphological condition of the overall skeleton and was defined as: Poor if their was widespread fragmentation of bone, severe erosion and post mortem damage, and a significant component of the trabecular bone was visible or lost. Moderate if there was some fragmentation of bone, marginal erosion and/or post mortem damage, and small areas of trabecular bone visible. Good if the bones were mostly whole and little to no erosion or post mortem damage.

Sexing was based on pelvic and cranial morphology using the criteria from Bass (1995), assessing nine attributes and classified as Male, Probable Male, Indeterminate, Probable Female and Female. Age at death was estimated using a number of methods, for adults these included Pelvic Morphology (Brooks & Suchey, 1990; Lovejoy et al., 1985) fusion of key epiphyses (Webb & Suchey, 1985) and tooth wear (Brothwell, 1981). For juveniles, age estmimates were calculated using epiphysial fusion (Shwartz, 1995), diphyseal length (Scheuer & black, 2000) and dental Eruption (Ubelaker, 1989). Trauma and Pathology was classified according to type and location according established osteological criteria (Aufderheide & Rodriguez-Martin, 1998; Ortner 2003).

Table 2.2: Burial Population From Towyn-Y-Capel:- Overall Condition, Preservation and Pathology.					
Skeleton	**Condition[1]**	**Preservation[2]**	**Age[3]**	**Sex[4]**	**Pathology[5]**
Sk1	Poor	Mod	adult	F	None
Sk2	Poor	Poor	?	?	Some porosity on cranial fragments
Sk3	Poor	Poor	subadult	?	None
Sk4	Good	Good	7-9	?	None
Sk5	Mod	Mod	subadult	?	None
Sk6	Mod	Poor	<15	?	None
Sk7	Poor	Poor	adult	?	Periostitis (?) in bilateral mandibular fossa, heavy calculus
Sk9	Poor	Poor	subadult	?	None
Sk10	Mod	Poor	adult	?	None
Sk11	Poor	Poor	adult	?	Possible cribra orbitalia?, periostitis+porosity rib tubercles, caries mandibular molars
Sk12	Poor	Poor	?	?	None
Sk13	Good	Poor	?	M?	Cranial and S1 fragments present only
Sk14	Mod	Poor	3.5-6.5	?	None
Sk15	Poor	Poor	1-2	?	None
Sk16	Poor	Poor	3-5	?	None
Sk17	Good	Good	17-23	F	Calculus
Sk18	Good	Good	16-20	?	Dental abscess, periodontitis?
Sk19	Poor	Poor	0-6m	?	None
Sk20	Good	Good	adult	F	Metopic suture (NB 38wk fetal remains in pelvic area)
Sk21	Mod	Mod	adult	M	None
Sk22	Mod	Poor	adult	?	None
Sk23	Poor	Poor	subadult	?	None
Sk24*	Good	Good	14-17	M?	None
Sk24*	Mod	Poor	adult	?	None
Sk25	Mod	Mod	adult	M	Slight lipping cervical vert
Sk26	Poor	Poor	adult	?	Lingual calculus
Sk27	Poor	Poor	subadult	?	MNI=2 (additional adult fragments)
Sk28	Poor	Poor	subadult	?	None

Table 2.2: Burial Population From Towyn-Y-Capel:- Overall Condition, Preservation and Pathology.					
Skeleton	**Condition[1]**	**Preservation[2]**	**Age[3]**	**Sex[4]**	**Pathology[5]**
Sk29	Mod	Good	1.5-6m	?	None
Sk30	Mod	Poor	adult	F?	Caries, calculus, blastic activity on Right transverse process of cervical vert
Sk31	Poor	Poor	subadult	?	None
Sk32	Mod	Good	38-40fw	?	None
Sk33*	Good	Mod	adult	F?	Some periostitis on leg
Sk33*	Mod	Mod	20-30	F	Osteoblastic lesion on R occipital/parietal
Sk34	Mod	Good	35-45	M	Abscess left maxilla PM1-M2
Sk35	Good	Good	15-25	M	Slipped epiphysis/pseudoarthrosis Right hip; Schmorl's nodes, scoliosis; Right proximal radius enlarged, subchondral cyst
Sk36	Poor	Poor	1-6	?	None
Sk38	Mod	Mod	40w-6m	?	None
Sk39	Poor	Poor	35-45	F?	Osteoma L parietal, caries
Sk40	Good	Good	2-5	?	Cribra orbitalia? periostitis postero-medial midshaft L femur
Sk41	Mod	Good	30-45	M	Osteitis L tibia/fibula
Sk42	Mod	Good	3-4	?	Enamel hypoplasia
Sk43	Mod	Good	45-60	F	Light fragile bones, thin trabeculae - osteoporosis? osteomyelitis? Right proximal fibula severe calculus 5-8mm thick, abscess Right mandible
Sk44	Mod	Good	1-2	?	None
Sk45	Mod	Good	1.5-2	?	None
Sk46	Poor	Poor	45-	M?	Displaced fracture/pseudoarthrosis L clavicle; Calculus
Sk47	Mod	Poor	adult	?	Periostitis bilateral fibulae
Sk48	Poor	Mod	20-25	F	Osteolytic activity MTT's 'cup and pencil'?
Sk49	Good	Good	40-45	M?	Schmorl's nodes, vertebral osteophytes; possible healed rib fracture; osteoarthritis - eburnation Right greater multiangular/MTC1; compression of T8 and L3 vertebrae?
Sk50	Good	Good	25-39	M	Spina bifida occulta s5-s3; systemic blastic activity; mild scaphocephaly, endocranial blastic lesions; dental abscess
Sk51*	Mod	Good	30-49	M	Cribra orbitalia, unfused sacrum s1-s2, osteophytes T10; periostitis anterior tibia, mandible abscess; subchondral cyst distal end proximal phalanx 1 (Right foot)
Sk51*	Good	Mod	adult	M	Schmorl's nodes thoracic, lipping thoracic and lower cervical verts
Sk52	Poor	Mod	25-45	F?	Cervical osteophytes - arthritic, dental overcrowding; metopic suture
Sk53	Good	Good	12-14	?	Metopic suture

Table 2.2: Burial Population From Towyn-Y-Capel:- Overall Condition, Preservation and Pathology.					
Skeleton	**Condition[1]**	**Preservation[2]**	**Age[3]**	**Sex[4]**	**Pathology[5]**
Sk54	Good	Good	45-60	F	Edentulous, slipped humeral epiphysis or joint disease? Spondylolysis L5
Sk55	Good	Good	17-25	F	None
Sk56	Good	Good	50-60	M	Sinusitis, dental anomalies
Sk57	Mod	Mod	25-35	?	Additional lumbar vertebra; rotated mandibular premolar, extensive periodontal disease
Sk58	Good	Poor	23-	?	Osteophytes distal fibula and tibia (only bones present!)
Sk59*	Good	Poor	23-	?	None (tibia only)
Sk59*	Poor	Good	adult	?	None
Sk60	Good	Good	35-45	F	Cribra orbitalia, thoracic osteophytes; midshaft healed parry-type fracture L ulna, Lt arm disused?; hammer deformity of 5th R toe , fusion mid-dist phals
Sk61	Good	Good	45-	M	Spinal fusion, compression fracture T12-L1, severe osteophytes; severe bilateral blastic activity at shoulder, arthritis? Hole in L ilium abscess Right maxilla PM1
Sk62	Mod	Good	3-6m	?	None
Sk63	Good	Good	27-35	M	Lumbarised S1, porotic hyperostosis? Drainage hole R occipital
Sk64	Mod	Mod	8-12m	?	None
Sk66	Good	Good	25-35	M?	Healed fracture R prox phals, osteoarthritis interphalangeal, fused toe 5 bilateral; spina bifida occulta
Sk67	Mod	Mod	25-35	M?	None
Sk68	Mod	Good	30-66	M	Compression of T7 and T9, osteophytes, blastic area medial inferior R patella-myositis?
Sk69	Good	Good	20-23	F	Metopic suture, unfused posterior arch C1
Sk70	Poor	Poor	25-35	F?	None
Sk71	Good	Good	1.5-3	?	None
Sk72	Good	Good	4.5-6	?	None
Sk73	Good	Poor	3-5	?	None
Sk74	Good	Good	34-40fw	?	None
Sk75	Good	Poor	2-2.5	?	None
Sk76	Mod	Poor	3-9m	?	None
Sk77	Good	Poor	25-	?	Squatting facets bilateral tibiae, subchondral cysts bilateral prox surface prox ph1
Sk78	Mod	Poor	0-6m	?	None
Sk79	Poor	Poor	adult	?	None
Sk80	Poor	Mod	12-18	?	Mild cribra orbitalia?, non-metric trait ossicles: lambdoid x3 (RHS), apical, sagittal

Table 2.2: Burial Population From Towyn-Y-Capel:- Overall Condition, Preservation and Pathology.					
Skeleton	**Condition[1]**	**Preservation[2]**	**Age[3]**	**Sex[4]**	**Pathology[5]**
Sk81	Good	Good	18-30	F	None
Sk82	Mod	Mod	45-	F	Osteophytes C1,C2, lumbarized T12, osteophytes on rib facets
Sk83	Good	Good	40-49	M	Sacralised L5 vertebra, compression and kyphosis T11; lytic/blastic activity R scapula, osteophytes bilateral fibula, calcaneus, ulna olecranon; fused proximal and intermediate phals
Sk84	Good	Good	0-2m	?	None
Sk85	Mod	Poor	17-25	F	Possible congenital absence molar 3
Sk86	Good	Good	0-6m	?	None
Sk87	Mod	Poor	23-	?	None
Sk88	Poor	Mod	35-45	F?	Compression L5 vert
Sk89	Poor	Mod	35-	M	Osteoporosis, osteophytes, compression L4+5, kyphosis L4+5 vert
Sk90	Poor	Poor	32-34fw	?	None
Sk91	Good	Good	0-6m	?	None
Sk92	Good	Good	3-9m	?	None
Sk93	Good	Good	60-87	F	Osteoarthritis and degenerative joint change, resorption of acetabulum
Sk94	Poor	Poor	?	?	Not examined
Sk95	Good	Good	3-6	?	None
Sk96	Good	Good	0-6m	?	None
Sk97	Good	Good	20-25	F	Osteophytes lumbar spine
Sk98	Poor	Poor	0-6m	?	None
Sk99	Mod	Good	60-	F	Osteophytes spine and scapula, porosity clavicle
Sk100	Poor	Poor	0-6m	?	None
Sk101	Mod	Mod	40-60	F	Osteophytes lumbar spine and femur, subchondral cyst humerus
Sk103	Good	Good	25-35	F	Spina bifida occulta, cyst distal tibia
Sk104	Mod	Poor	25-	?	None
Sk106	Good	Poor	35-45	M?	None
Sk107	Poor	Poor	0-6m	?	None
Sk108	Mod	Good	55-60	F	Spinal osteophytes, abscess mandible; displaced fracture Ldistal forearm, pseudoarthrosis (wrist), 16mm humerus length difference.
Sk109	Mod	Mod	1.5-2.5	?	None

Table 2.2: Burial Population From Towyn-Y-Capel:- Overall Condition, Preservation and Pathology.

Skeleton	Condition[1]	Preservation[2]	Age[3]	Sex[4]	Pathology[5]
Sk110	Poor	Poor	adult	?	Two rib fragments and skull frags only
Sk502	Mod	Poor	1.5-2	?	None
Sk506	Mod	Poor	15-24	?	None
Sk508	Mod	Mod	adult	F	Spinal fusion, dental abscess, thickened cranial diploe
Sk510	Mod	Poor	<15	?	None
Sk511	Mod	Good	adult	F	Remodelled rib neck fracture, cribra orbitalia, porous atrophied mandibular condyle; calculus, periostitis lower long bones
Sk512	Mod	Poor	3-5	?	None
Sk513	Mod	Poor	adult	?	Calculus
Sk514	Mod	Poor	<16	?	None
Sk515	Good	Poor	adult	M	None
Sk516	Good	Good	adult	M	Cribra orbitalia, vertebral joint disease
Sk517	Mod	Mod	15-18	?	Porosity in both orbits- possible cribra orbitalia?
Sk518	Poor	Poor	adult	?	None
Sk520	Poor	Mod	3-6	?	None
Sk521	Good	Good	35-55	M	Age-related joint change?

This table lists all the skeletons recovered from the Towyn-Y-Capel Site (GAT1746) and their associated condition, preservation, sex age and Pathology. These are given in standard anthropological notation.. Highlighted rows correspond to skeletons with successful sequencing as indicated in §5. [1]Condition:.- the degree of fragmentation and destruction of bone. [2]Preservation: - the completeness of the skeletal assemblage[3] Age:- as deduced from available anthropological evidence, un-noted number correspond to age in years, ages >1 year shown by m-months & w-weeks. [4] Sex, given as M-Male, F-female &?-uncertain. [5]Pathology, as determined from osteological examination and anthropological convention. [*]Multiple Interpretations of Skeletal Pathology is given for the same skeleton where inconsistent skeletal element interpretation (preservation, condition, age or sex). Abbreviations used: frag- Fragments, R/L- Right/Left vert-vertebrae prox-proximal, int-intermediate phal-phalanges.

2.3 Laboratory Material and Methods

2.3.1 Sample Examination and Selection

All skeletons, where practicable, were sampled for appropriate patellae and teeth for genetic investigation. Some skeletal assemblages were missing both or either appropriate patella or teeth samples, and there were some samples that, due to degradation or physical disruption, were not associated with a skeletal assemblage and were therefore labelled as unknown (ie UkA). These latter cases were used predominately for preliminary studies.

Bones

Patellae varied widely in their states of preservation, reflecting the variability in the site itself from one burial to another. A brief overview of the condition, preservation and skeletal biology of the buried site population is given in table 2.2. In the laboratory, all efforts were made to minimise sample interaction with contaminants. Samples were stored under clean asceptic conditions and were not exposed to surface or human physical interactions. They were manipulated in a dedicated clean laboratory in order to reduce contamination of the samples. Samples were handled asceptically with sterile implements. Only this carried out the handling and subsequent manipulations, with double gloved hands, fully covered body and a face mask.

Samples were brushed free of excess soil and sand and rinsed lightly in a sequence of washes comprising: sterile ddH_2O, sodium hypochlorite solution, sterile water, and the detergent, Microsol, for no longer than 10 min each. After these washes, samples were rinsed with water again and finally with ethanol before being left to dry.

Samples were digitally photographed (Canon Powershot Pro 90 IS and Canon EOS 300D SLR with Macro Lens) for later non-tactile osteological analysis, and to comprehensively record all the dental material before their use in destructive sampling techniques. Scale photographs were taken of all the patellae on both the anterior and posterior sides in order to record their size, shape, anatomical orientation and gross condition. Before and after photography, samples were treated to 10 min of irradiation under a combined UV lamp and transilluminator (254 nm) in a UV sterilisation cabinet (Geneflow). Before grinding or sectioning, samples were further irradiated under a combined UV lamp and transilluminator (254 nm) for 20 min on both the anterior and posterior sides of the patella. This step was undertaken prior to the bone being crushed to minimise contamination. Osteological material was kept at ambient temperature as a complete bone structure, but after grinding, bone powder was kept at -20^{o} C in 2 ml screw top cryotubes in aliquoted and preweighed aliquots of 300-500 mg.

Teeth

Archaeological tooth samples were taken directly from skeletal assemblages, and where possible from the mandible or maxillae, closely associated with the skeletal assemblages. Teeth that were associated with a skeleton but not bound (ie. found loose in the same grave) were also collected. Teeth were selected preferentially in the following order: 3rd molar, 2nd molar, 1st molar and then others. Molar teeth were preferentially selected due to their large volume, both of the pulp chamber (for genetic analysis), enamel (for the inorganic analysis) as well as the greater structural preservation generally found with these teeth due to their later development in adults.

Modern tooth samples were used both as controls and to optimise later protocols, and were primarily selected according to availability, integrity, lack of discoloration, preservation and to the lack of caries. Archaeological tooth material was subjected to a slightly different treatment to minimise contamination with exogenous material. Samples were brushed free of excess soil and sand, rinsed lightly with sterile ddH2O, washed for 10 min with sodium hypochlorite (bleach) and rinsed with sterile water again, before being washed with the detergent, Microsol (Anachem), for no longer than 10 min. Finally, samples were rinsed again with water and then with ethanol and left to dry.

Samples were digitally photographed (Canon Powershot Pro 90 IS and Canon EOS 300D SLR with Macro Lens with 2x 10 watt Halogen spotlights) for later non-tactile osteological analysis, in order to record the gross condition of the teeth before the use of destructive sampling techniques. Scale Photographs were taken of all teeth on occlusal, mesial, distal, buccal and lingual sides, in order to give a complete representation. Before and after any bone investigation samples were treated with 10 min of irradiation under a combined UV lamp and transilluminator (254 nm) in a UV sterilisation cabinet (Geneflow). Before grinding/sectioning the samples were further irradiated under a combined UV lamp and transilluminator (254 nm) for 20 min simultaneously along the mesial-distal orientation. This step was undertaken just prior to the tooth being crushed using a nitrogen mill grinder. Intact dental material was stored at ambient temperature, but once samples were ground, they were stored at -20^{o}C as tooth powder in 2 ml screwtop cryotubes in pre-weighed aliquots of 300-500 mg.

2.3.2 Gross Preservation of Towyn-Y-Capel Samples

In the field of anthropology, it is generally accepted that the morphological and histological preservation of calcified material is essential for inorganic and organic chemical analyses to be undertaken (Haynes *et al*., 2002). Accordingly, evaluation of the condition and state of preservation of the individual skeletal units, which were primarily patellae and teeth, was undertaken prior to destructive genetic and chemical analysis, in order to understand if not select appropriate samples.

The preservation of skeletal material was evaluated by visual examination of the high-resolution images captured under clean room conditions and, where necessary, by additional visual conformation of skeletal elements in a UV sterilisation cabinet (§ 2.2.1). The condition of skeletal material was evaluated using a grading system, 'The gross preservation index (GPI)', based on the specific index introduced by Haynes *et al*., (2002). The morphological condition of bones and teeth were determined by the visual inspection of calcified tissue and graded into four categories that ranged from poor condition (GPI 1) to excellent condition (GPI 4). The criteria used in this grading analysis are described in table 2.3 for patella and in table 2.4 for teeth. In order to verify the GPI index and to correct for the highly subjective nature of the criteria utilised, the images of skeletal samples were independently graded by multiple unbiased observers (n=11) using this index. From these data, normalised values for the GPI of individual skeletal elements within the population were determined.

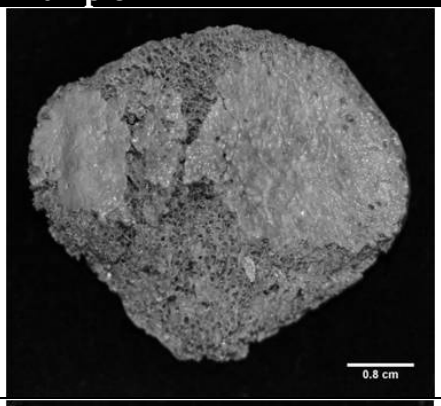
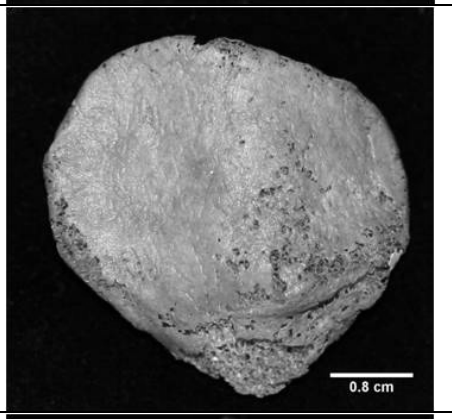
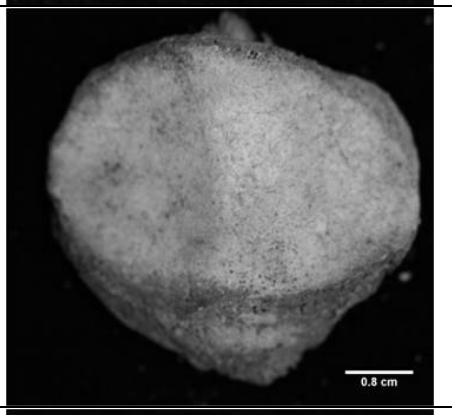
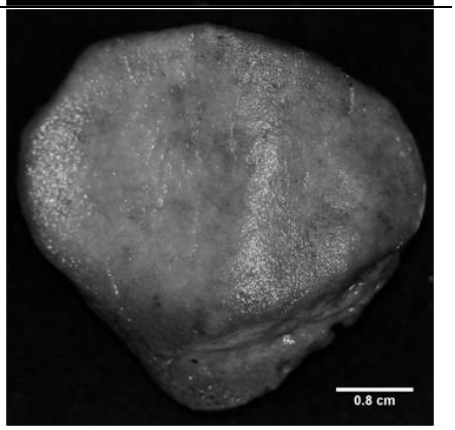
GPI	Description	Example
1	Preservation is "**Poor**". Surface is dull and rough. Surface id friable. Flaky and inner layers of bone are exposed. Breakages and exposed bone are crumbly, brittle and indistinct. Material feels light and hollow.	
2	Preservation is "**Fair**". Surface without shine, texture rough and grainy. Extensive pitting of the surface, or small areas of the surface missing (<1 mm patches). Broken edges or exposed bone may be crumbly.	
3	Preservation is "**Good**". Surface is shiny and smooth, but texture is slightly grainy. Surface may appear pitted under dissecting microscope (10 x magnification), but not exceeding 40% of the field of View. Breakages or exposed inner surfaces not crumbly.	
4	Preservation is "**Excellent**". Almost pristine material. Surface is shiny, smooth, greasy and without pitting. Material is relatively heavy and dense. Chips or breakages are clean and distinct.	

Figure 2.1: Gross Preservation Index (GPI) used to Assess Archaeological Bone, specifically Patella from Towyn-Y-Capel* (*Adapted from Haynes *et al.*, 2002)

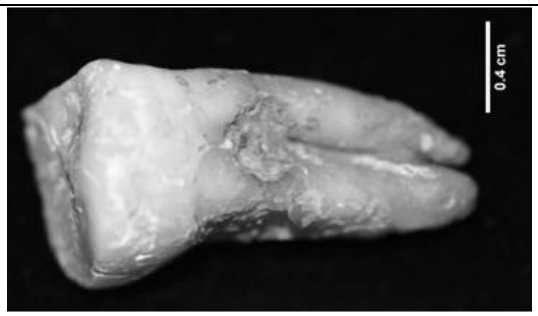
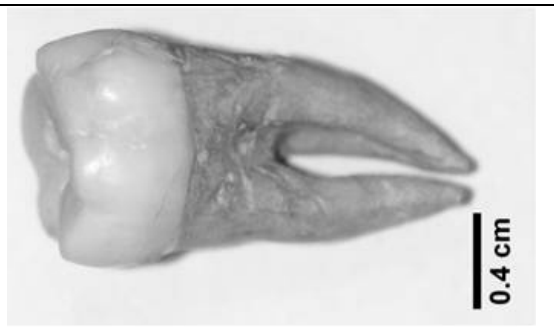
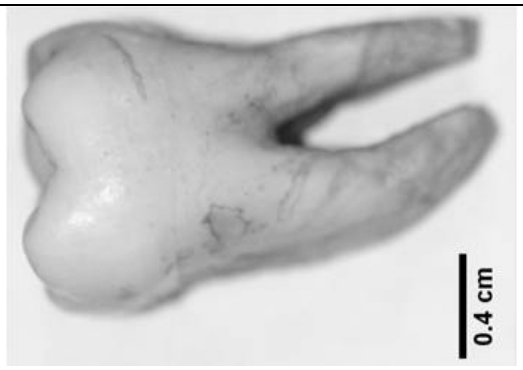
GPI	Description	Example
1	Preservation is "**Poor**". Surface is dull and rough. Enamel cracked and or flaking. Severe staining, opaque and chalky. Severe brittleness of dentin (loss of structure across the tooth) and flaking. Material feels light and hollow.	
2	Preservation is "**Fair**". Surface without shine, texture rough and grainy. Loss of gloss in the enamel. Material is translucent. Chalky or opaque dentin near root surface. Stains in enamel, some flaking and dentin is softening or slightly brittle. Broken edges or exposed bone may be crumbly.	
3	Preservation is "**Good**". Surface is shiny and smooth, but texture is slightly discoloured. Enamel still glossy, maybe a little translucent. Some Discolouration. Breakages or exposed inner surfaces not crumbly.	
4	Preservation is "**Excellent**". Almost pristine material. Surface is shiny, smooth, enamel glossy but not translucent Material is relatively heavy and dense. Chips or breakages are clean and distinct. No Discolouration.	

Figure 2.2: Gross Preservation Index (GPI) used to Assess Archaeological Teeth from Towyn-Y-Capel[*] (*Adapted from Haynes *et al.*, 2002)

2.3.3 Estimating Structural Preservation in Ancient Teeth using Quantitative Light Fluorescence (QLF) in Whole Teeth

Quantitative Light Fluorescence (QLF), is a recently developed technique, which is based on the autofluorescence of teeth when bathed in blue-green light (488 nm), and is used in clinical dentistry to evaluate the degree of dental demineralization caused by disease. In a novel approach to the field of molecular archaeology this technique was used for the non-destructive assessment of the degree of demineralization of ancient teeth. These results could then be used as a measure of the inorganic molecular and structural condition of the teeth. In the work here, in order to determine the degree of demineralization of ancient teeth, the relative fluorescence intensities of the ancient teeth were compared to those of modern teeth, which acted as a control. To facilitate these comparisons, a calibration curve of relative fluorescence versus level of demineralization was constructed for control teeth using simultaneous X-ray and QLF experiments with modern bovine samples.

Sequential Quantitative Determination of the Structural Condition of Demineralized Samples by using Whole Tooth Autofluorescence, X-ray Transmittance and Calcium Release.

Numerous teeth were used in trial experiments to determine the approximate the rate of demineralization and structural integrity of modern control teeth. This work was limited, however, by the scarce availability of modern human teeth, particularly in good condition. In place of human teeth whole intact bovine incisors were used for comparison. These teeth have a similar size, weight and dentin-enamel ratio to human molars and are generally considered to be a suitable model for these latter teeth. Accordingly, bovine incisors were used as control teeth for this research project. These teeth were cleaned and surface polished using pumice powder and a high-speed hand held brush. Complete and intact teeth were then placed in rotating Falcon tubes (50 ml) containing 15 ml of EDTA (0.5 M) and sequentially demineralised over a period of 22 days through regular substitution of the EDTA solution. The degree and rate of demineralization of teeth were determined by three concomitant analyses: the detection of relative auto-fluorescence intensity using the QLF system, X-ray transmittance and quantification of the calcium released from the teeth into solution by Atomic Adsorption Spectroscopy. The QLF system measured the intensity of the auto-fluorescence of teeth under blue light by recording an image using a filtered microvideo and performing computerized analysis of this image utilising the QLF Inspektor Pro image acquisition software, all as described by the manufacturer (Inspektor™ version 1.99, Amsterdam, NL).

X-ray transmittance analysis was performed using a custom made dental desktop X-ray emitter box with standard settings and distance, and an exposure time of 2 min for a Polaroid

photographic plate upon which both the control and sample teeth had been set. Calcium released into solution from teeth was calculated from the discarded EDTA solutions and measured on an ATI/UNICAM 929 Atomic Absorption Spectrometer (AAS) using Flame Absorption setup to detect calcium (422.7 nm with a 0.5 ηm bandpass). The solution was volatised for 2 sec over an air/C2H2 Flame at a rate 1.2 l/min. Initial concentration curve set for calibration using 5 standards between 1-10 mg/L. Readings taken of sample solution at 1/2000 dilution due to high calcium concentrations.

Image analysis for both QLF and X-ray analysis was performed using image J software (version 1.60-10 Biomedical Image Analysis Software, National Institutes of Health, USA), which determined the mean fluorescence and transmittance of the exposed enamel surface of each tooth analysed, normalised against the untreated control tooth.

Quantitative Determination of the Structural Condition in Towyn-Y-Capel Samples by using Whole Tooth Autofluorescence

After decontamination, ancient teeth were visualized under the fluorescent QLF Research system as described above, both before and after dental cleaning. This cleaning procedure involved the removal of surface inclusions, stains and deleterious material by dental scraping and pumice stone polishing. Relative fluorescence intensity was then determined utilising the QLF Inspektor system and image J software. Through extrapolation against the sequential demineralization model, an indication of the loss of the inorganic faction was calculated.

2.3.4 Taphonomical Investigations:- Histology

Although the gross condition of bones could provide much information on their state of fossilisation, the important parameters in this respect were the integrity of the organic and inorganic factions. Contemporary theories regarded both the structural and cellular integrity of bones as being good indicators of molecular preservation and thus, it was the histological condition of the bone or tooth that could indicate the difference between good molecular condition and otherwise (Guarino et al., 2000). Unfortunately, however, the histological examination of calcified tissues was a complex and destructive process. The Towyn-Y-Capel population was an archaeologically sensitive with limited availability for skeletal material designated for destructive analysis. It was therefore, considered unadvisable to undertake a comprehensive histological analysis of the site's population. As an alternative strategy, a generalised investigation was performed on loose non-human material found at the site.

This material was predominately scattered faunal bone fragments that were discovered at the site in similar stratigraphy to the skeletal population and were considered to have

undergone similar *post-mortem* degeneration in relation to chemical, biological and physical environmental factors.

Table 2.1 shows the bone and teeth fragments that were available to study along with putative identification. The samples included: bird femurs, long bone fragments from either pig or sheep, and some non-identified animal bone. For comparison, modern samples of similar calcified material from rabbit (*Oryctolagus cuniculus*) and chicken (*Gallus gallus domesticus*) sources were also prepared for histological investigation. The specific samples used for this histological investigation, including modern controls, are given in table 2.8.

Modern Sample Digestion

Prior to the preparation of modern bone samples for histological mounting, the removal of excess non-calcified tissue was performed and samples boiled under pressure for 1-1.5 hours to remove remaining soft tissue. These bones were then oven dried at 120^{o} C for 5 min. Care was taken throughout this procedure avoid "scoring" the bone or damaging the material in any way, both in the case of modern and ancient samples.

Preparation of Modern and Ancient Samples

Sections of cortical diaphysis bone were cut that were 3-4 mm in thickness using a fine hacksaw, and initially polished with fine, wet and dry glass slide mounted sandpaper (P400 grade sandpaper, mounted using cyanoacrylate glue). Samples were then cleaned with sterile water and then detergent followed by sterile water rinse, and finally, with an ethanol (90% v/v) rinse. Post rinsing, the sample was fixed to a glass slide using cyanoacrylate glue) and left for 2 hours to harden. To reduce sample thickness to *circa* 1 mm, the mounted sample was ground using fine grade dry glass mounted sandpaper (P180 grade sandpaper and mounted on a glass slide as before). Further grinding was performed using wet sandpaper (P280 grade sandpaper and mounted on a glass slide as before) until the sample was transparent when observed through a bright field microscope or oblique lighting. In between these grinding steps, samples were regularly cleaned using sterile water, detergent followed by sterile water and ethanol 90% (v/v). A final polishing step was undertaken using wet sandpaper (P400 mounted on a glass slide), followed by a cleaning step. This procedure was adapted from Maat *et al*., (2001). Histological slides were analyzed using a Leica DMZ-R upright compound fluorescence microscope and images were captured by a Nikon D50 using brightfield and polarised light microscopy. Images were compared to a standardised histological preservation index (HPI), adapted from Haynes & colleagues (2002), analogous to the GPI.

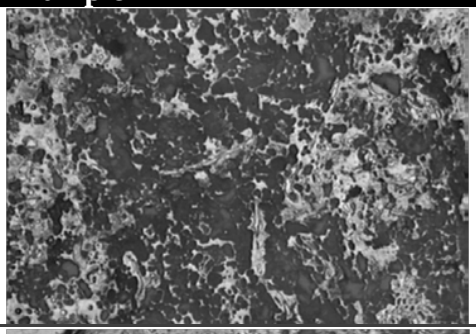
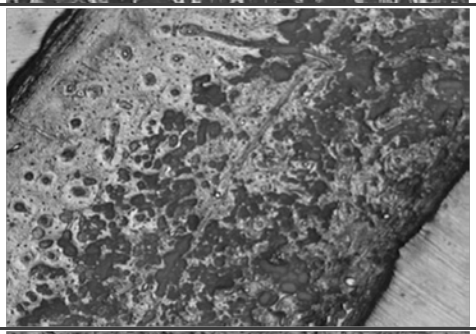
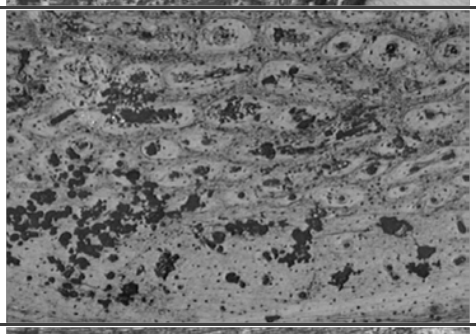
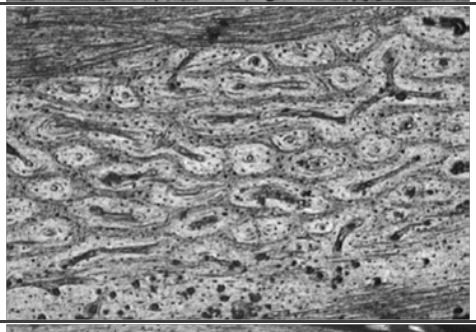
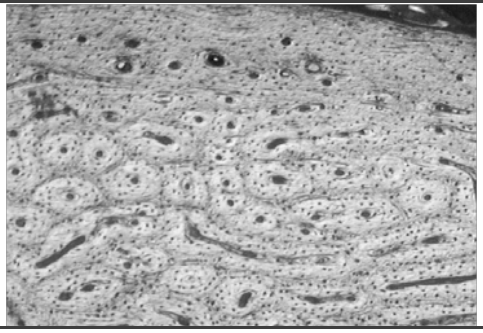
HPI	Description	Example
1	No structures, except possibly the surface lamellar layers are visible. **(Poor)**	
2	There are small areas of organised structures visible (~25% of the bone). There are extensive areas of destruction. **(Moderate)**	
3	Patchy preservation. Areas of damage leave irregular holes. Structures are visible in at least 50% of the bone. **(Fair)**	
4	All structures are clearly visible in at least 80% of the bone. **(Good)**	
5	Virtually indistinguishable from modern bone. At least 95% preservation. **(Excellent)**	

Figure 2.3: Histological Preservation Index (HPI) used to Assess for Archaeological Bones material from Towyn-Y-Capel.* (Adapted from Haynes *et al.*, 2002)

Table 2.3: Specimens used for Histological Analysis.			
Modern Specimens			
Slides	**Sources**	**Bone Types**	**Observations**
A	Pigeon	Femur Long Bone Diaphysis	N/A
B	Pigeon	Femur Long Bone Diaphysis	N/A
C	Rabbit	Long Bone	N/A
D	Rabbit	Long Bone	13
E	Rabbit	Long Bone Epiphysis	4
F	Rabbit	Long Bone Diaphysis	6
G	Rabbit	Long Bone Diaphysis	4
H	Rabbit	Not Determined	5
I	Chicken	Femur Long Bone Diaphysis	7
J	Chicken	Femur Long Bone Diaphysis	6
K	Chicken	Femur Long Bone Diaphysis	10
L	Chicken	Femur Long Bone Diaphysis	6
M	Chicken	Femur Long Bone Diaphysis	9
Ancient Specimens (Towyn-Y-Capel)			
Slides	**Putative Sources**	**Bone Types**	**Observations**
N	Unknown fauna	Unidentified Bone Fragment	4
O	Chicken	Femur Long Bone Diaphysis	23
P	Chicken	Femur Long Bone Diaphysis	17
Q	Chicken	Femur Long Bone Diaphysis	13
R	Unknown fauna	Unidentified Bone Fragment	6
S	Unknown fauna	Unidentified Bone Fragment	8
T	Unknown fauna	Unidentified Bone Fragment	6
U	Unknown fauna	Unidentified Bone Fragment	?
V	Pig or sheep	Long Bone Diaphysis	10
W	Unknown fauna	Unidentified Bone Fragment	8

Slide designation corresponds to Sample Slide Number used for investigation. Modern samples were digested before grinding and mounting as discussed in the text. Pigeon- *Columba livia* f. *domestica*, Rabbit-*Oryctolagus cuniculus*, Chicken-*Gallus gallus domesticus.*

2.4 Results and Discussion

2.4.1 Sample Recording and Photography

Teeth and bones samples from Towyn-Y-Capel archaeological site were measured and photographed as described in section 2.21, an example of which is given in figure 2.1. The entire collection of patella and teeth is kept as a digital record (Appendix 2.1 and 2.2). These high resolution images provide permanent records for future odontological analysis and allow the determination of the GPI without the need for further laboratory handling thus minimising possible contamination. From a skeletal perspective cist burials (Sk81, Sk83, Sk84, Sk86, Sk87, Sk89, Sk90, Sk91, Sk92, Sk93, Sk95, Sk96, Sk97, Sk99, Sk100 Sk102, Sk105, Sk 108 & Sk 109) were mainly well preserved, and in good to moderate condition. The later dug burials were polarized between good preservation and very poor preservation, due in main to the site erosion, and a uniform distribution in condition between Poor, moderate and good. The preservation and condition had profound effects on the selection of appropriate biochemical samples, predominantly because of the availability of the sampled skeletal element, but also necessary integrity.

2.4.2 Gross Preservation of Towyn-Y-Capel Samples.

The gross preservation of the sampled patellae and teeth from the Towyn-Y-Capel population (Figure 2.4) and assessed according to the criteria of Figures 2.1 and 2.3, respectively, is shown are in figures 2.5 and 2.6 collectively, and individually for both bone and teeth components in table 2.7. The small sample sizes (n=54) and number of the observers (n=10), combined with the subjective nature of this form of analysis emphasises that the GPI only provides a guide to the state of the tissue preservation, especially in light of more specific molecular techniques such as protein chemistry and genetics. Nonetheless, the technique does allow for a fast, simple and effective preliminary tool for estimating the preservation of skeletal elements. It has the advantages that it does not compromise the sample for subsequent retrieval of sensitive downstream information whilst keeping a permanent record of the sample for future verification and analysis.

GPI analysis showed that in the sample sets, the condition of over 60% of the bones and 50% of the teeth were classified as 2 on the GPI scale, or good. In teeth over twice as many samples were designated as being 3 on the GPI scale, or fair, as compared to the bone samples. However, both sample sets show elements at the level of *circa* 20% that were rated as GPI 1, or poor, and neither sample set had items in the excellent range, or 4 on the GPI scale.

These results could be interpreted as showing that the preservation of the bone elements across the whole population was good. However, the fact that the range of skeletal elements rated as GPI 1, or poor appears to be unrepresented might not be truly indicative of the population, as the selection procedure for samples would have skewed the statistics of GPI analysis. For example,: the teeth and bones that may be rated as GPI level 1 are most likely those in a brittle or partially non-intact state, and therefore, either from lack of association or rejection in the selection procedure have not been included in the sample population.. This would be particularly relevant in the case of teeth where non-intact teeth were not selected due to the higher likelihood of organic and inorganic contamination.

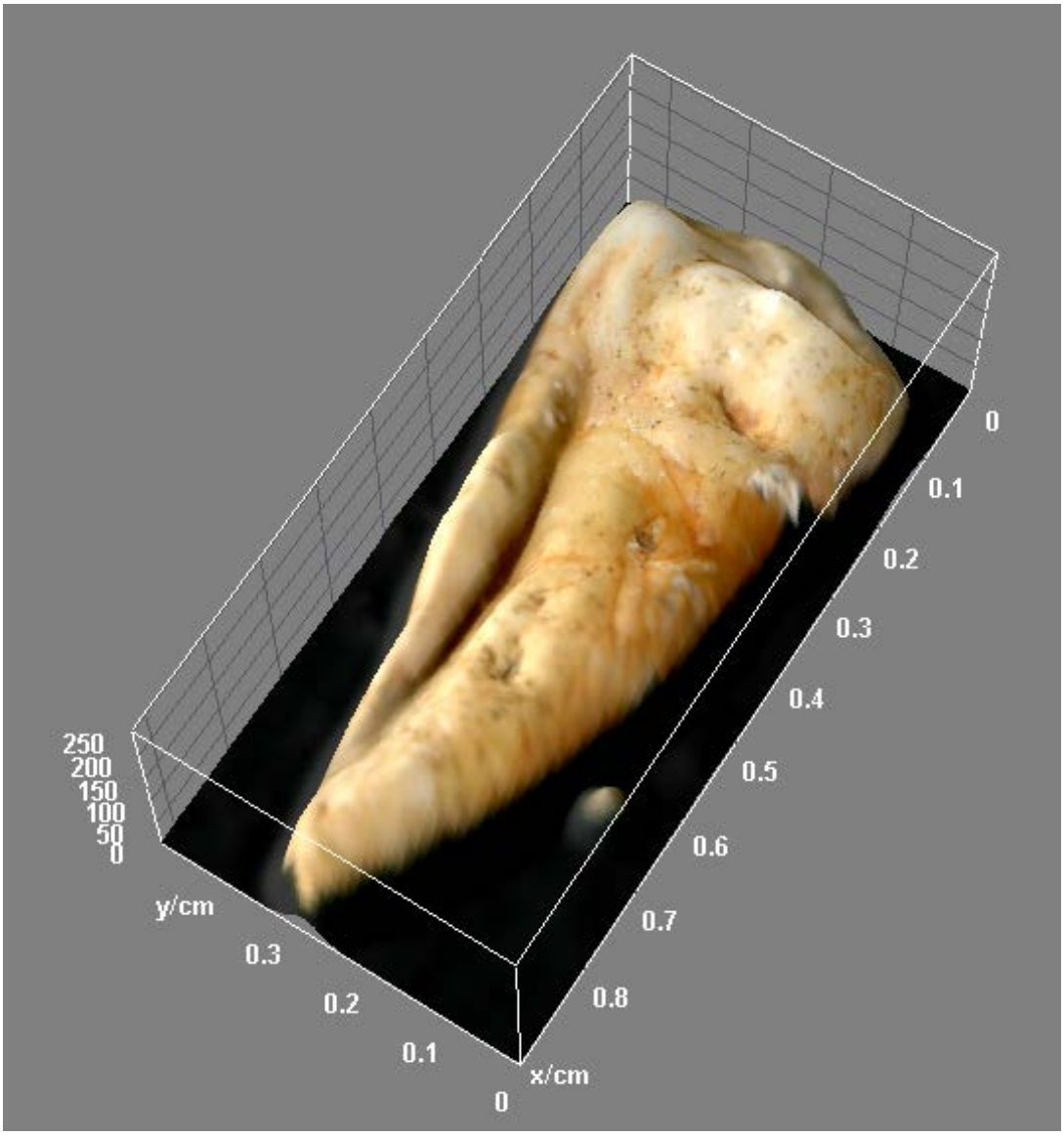


Figure 2.4: Ancient Tooth (Sk103) Showing Measurements and Pixel Light Intensity. Tooth visualized in depth and according to scale using image J software. All teeth were recorded digitally from 3 axis in order to allow for a reconstruction should it be necessary for later verification.

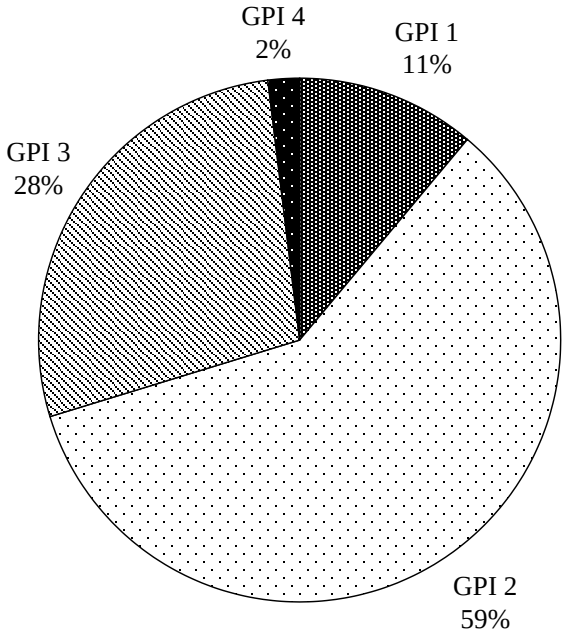


Figure 2.5 Gross Preservation Index Distribution of Bone (Patella) Sampled from Towyn-Y-Capel Population. The Pie chart above shows the distribution of the Towyn-Y-Capel population (n=54) with regards to the bone preservation of the sampled patella, given as GPI, where GPI 1- Poor, GPI 2-Fair, GPI 3-Good, GPI 4 –Excellent.

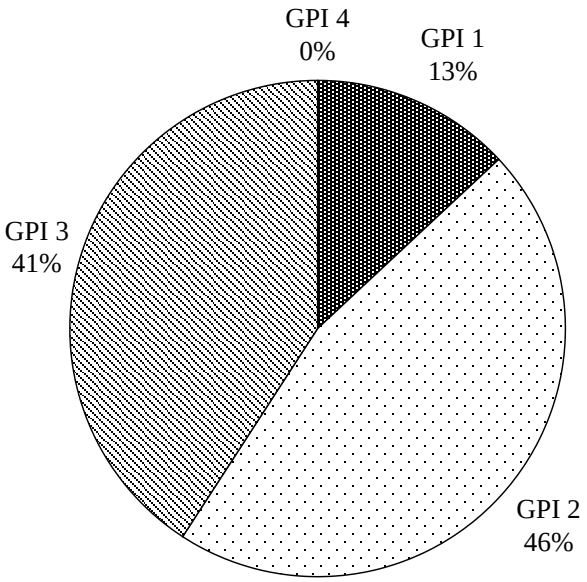


Figure 2.6 Gross Preservation Index Distribution of Teeth (Predominantly Molars) Sampled from Towyn-Y-Capel Population. The Pie chart above shows the distribution of the Towyn-Y-Capel population (n=54) with regards to the tooth preservation of the sampled molars, given as GPI, where GPI 1- Poor, GPI 2-Fair, GPI 3-Good, GPI 4 –Excellent.

Table 2.4: List of Skeletal Patella &Teeth GPI from Towyn-Y-Capel							
No	**Skeleton**	**Patella GPI**	**Teeth GPI**	**No**	**Skeleton**	**Patella GPI**	**Teeth GPI**
1	Sk001	2	2	43	Sk068	2	1
2	Sk004	N/A	3	44	Sk069	2	2
3	Sk011a	N/A	1	45	Sk070	N/A	2
4	Sk011b	N/A	2	46	Sk071	N/A	3
5	Sk013	N/A	3	47	Sk072	N/A	3
6	Sk016	N/A	3	48	Sk080	3	2
7	Sk017	2	3	49	Sk081	N/A	3
8	Sk018	N/A	3	50	Sk082	N/A	1
9	Sk020	2	3	51	Sk184/84	3	N/A
10	Sk021	N/A	3	52	Sk085	N/A	2
11	Sk022a	1	N/A	53	Sk087	N/A	3
12	Sk022b	1	N/A	54	Sk088	1	2
13	Sk024	N/A	2	55	Sk089	N/A	2
14	Sk025	1	2	56	Sk093	2	N/A
15	Sk028	N/A	3	57	Sk099	N/A	1
16	Sk030	N/A	3	58	Sk101	2	N/A
17	Sk033	3	3	59	Sk102	3	N/A
18	Sk033b	4	N/A	60	Sk103	2	2
19	Sk034	2	2	61	Sk104	2	N/A
20	Sk035	2	2	62	Sk105	2	N/A
21	Sk041	3	2	63	Sk106	N/A	3
22	Sk043	2	2	64	Sk107	N/A	1
23	Sk047	2	N/A	65	Sk108	2	1
24	Sk048	2	2	66	Sk502	N/A	2
25	Sk049	3	N/A	67	Sk506	N/A	3
26	Sk050	2	N/A	68	Sk508	2	3
27	Sk051	2	3	69	Sk508b	2	N/A
28	Sk051b	3	N/A	70	Sk510	2	N/A
29	Sk052	N/A	2	71	Sk511a	2	N/A
30	Sk053	2	2	72	Sk511b	1	3
31	Sk054	3	N/A	73	Sk513a	N/A	2
32	Sk055	2	N/A	74	Sk513b	N/A	2
33	Sk056	2	3	75	Sk514	2	N/A
34	Sk057	N/A	2	76	Sk516	3	3
35	Sk059	3	N/A	77	Sk517	N/A	3
36	Sk060a	3	N/A	78	Skuk0	1	N/A
37	Sk060b	2	N/A	79	Skuk1	3	N/A
38	Sk063	2	2	80	Skuk2	3	N/A
39	Sk064	N/A	2	81	Skuk3	3	N/A
40	Sk066	2	N/A	82	Skuk4	3	N/A
41	Sk067a	2	2	83	Skuk5	2	N/A
42	Sk067b	N/A	1	84	Skuk6	2	N/A

GPI assessed according to figures 2.1 and 2.2, by 11 different observers. Some bones and/or teeth were not available for all consequent Skeletons, absent teeth or patella listed as N/A – Not Available.

2.4.3 Quantitative Determination of the Structural Condition in Towyn-Y-Capel Samples by using Whole Tooth Autofluorescence

The QLF technique provided some very interesting insights into the condition and preservation of teeth. In general, there were large differences in the autofluorescence of ancient samples after cleansing primarily due to the removal of loosely associated surface detritus and stains. After the cleansing, ancient samples showed generally strong fluorescence under the QLF, particularly in the case of the enamel crowns of the molars studied. However, although high, these levels of fluorescence did not quite reach the fluorescence intensity of modern teeth, as can be seen in representative images of the modern premolar and Towyn-Y-Capel molar in table 2.4. Of particular note is the difference between the fluorescence of the dentin and the enamel in ancient teeth compared to modern teeth. The results used for demineralization comparisons and integrity were therefore from the dental crown of the tooth, or the enamel section, and not the whole tooth for ease of uniformity due to the variety of sample sizes and proportions of enamel.

2.4.4 Sequential Tooth Demineralization Model to Determine Correlation of Auto-fluorescence and Structural Integrity.

Both the QLF intensity and X-ray transmittance values of bovine incisor teeth were shown to be similar to expected values for modern human molar teeth, and hence, the former teeth were used as demineralization models. The results in figure 2.9 show the rate of demineralisation of ten teeth under the same condition, which were monitored by loss of relative fluorescence and X-ray transmittance and the data normalised against the control in both cases. Variations in the QLF intensity and X-ray transmittance values over the period of assay were also taken into consideration and results normalised against the controls over the entire 25 day period. It can be clearly seen in figure 2.9, that there is an exponential decline in both the fluorescence and X-ray transmittance values of samples, with a strong correlation between data curves. As an example, both data curves indicate that over 50% of the estimated mineral content of samples has been removed within 2 days, which can be seen in figure 2.9 to closely correlate with the decrease in calcium levels, which fell from 14 to 4 g/L in 2 days.

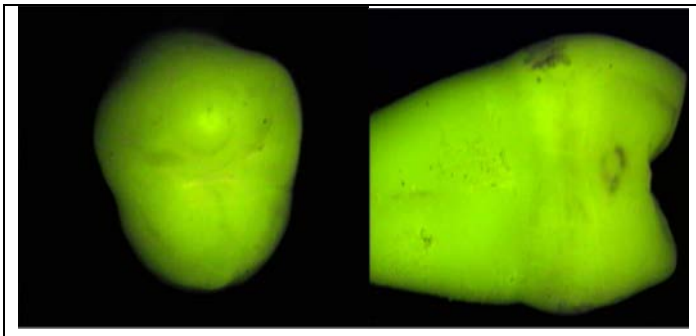
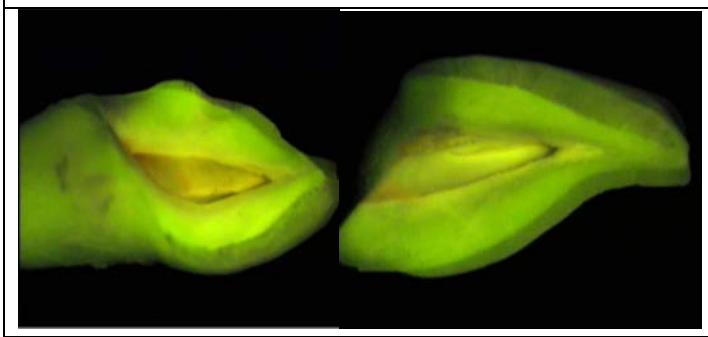
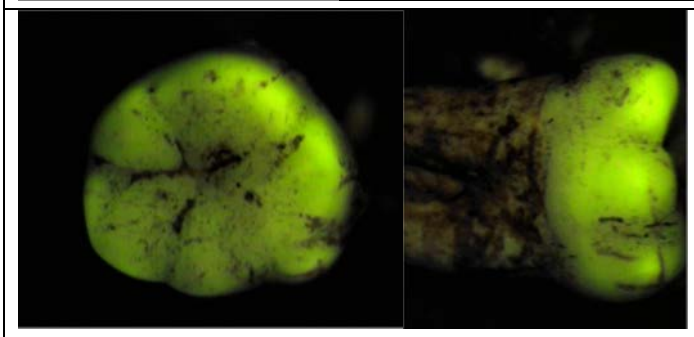
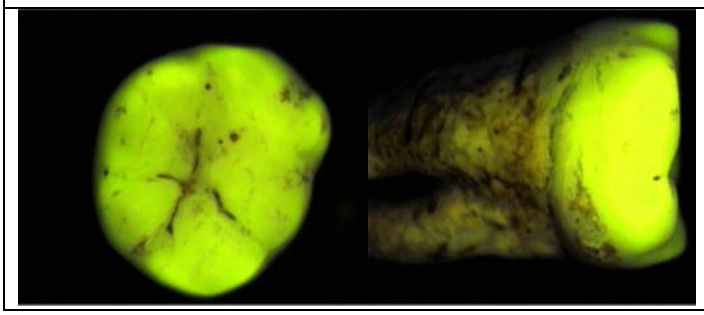
	A) Modern Human Tooth Premolar QLF Control Tooth Uncleansed Crown and lateral view
	B) Section through a Modern Human Incisor (modern Control) Lateral and cut section view Shows inside crown, From the enamel on the outside, the Dentin Enamel Junction (DEJ), dentin, and Dentinal Pulp Junction (DPJ) & pulp cavity from both views
	C) Ancient tooth from Towyn-Y-Capel (Sk26 Lower RM_3) Uncleansed Crown and lateral view
	D) Ancient tooth from Towyn-Y-Capel (Sk26 Lower RM_3) Cleansed Crown and lateral view

Figure 2.7: QLF in a Modern Control Tooth (A) including tooth Section (B) and Early Medieval Teeth and Pre (C) and Post (D)Cleansing

This figure shows the qualitative difference in the QLF intensity from modern and ancient teeth (A & D), and the difference in the fluorescence through different sections (enamel/dentin) of the tooth structure (B). And the noticeable difference in QLF intensity between the cleansed and cleansed ancient teeth (C & D). Of particular note is the loss of fluorescence in the Dentin of ancient teeth as seen in C and D.

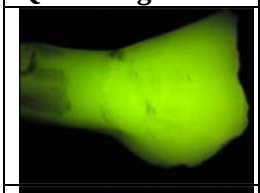
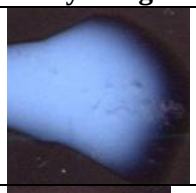
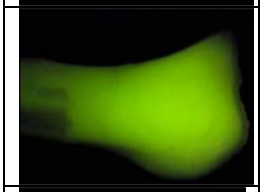
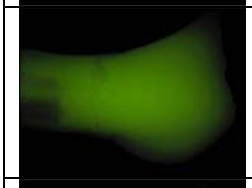
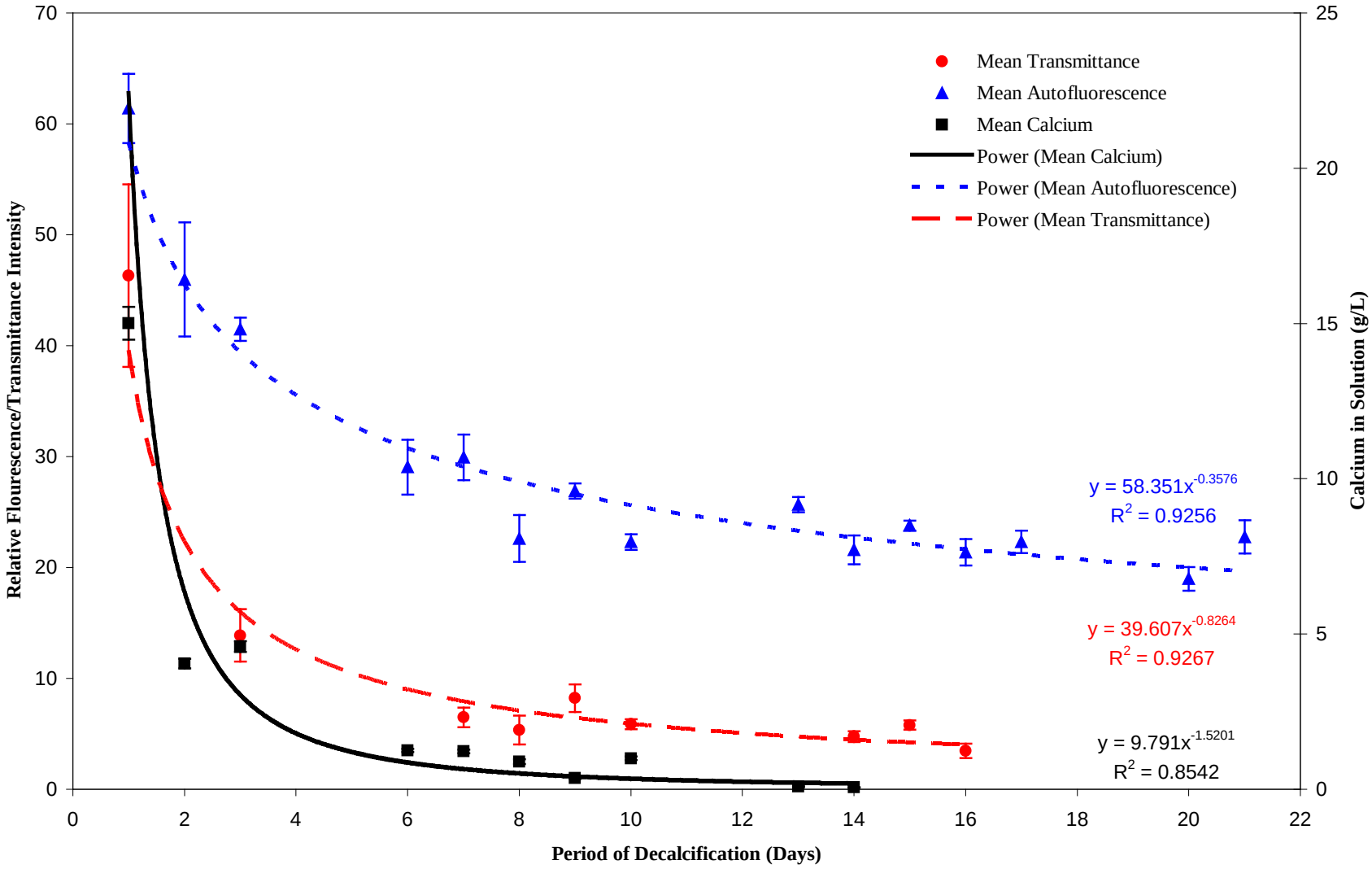
QLF Image	X-ray Image	Period	Calcium in Solution
		Day 1	13.835 g/L
		Day 3	4.856 g/L
		Day 8	0.988 g/L
		Day 10	0.640 g/L
		Day 14	0.093 g/L
		Day 16	N/A

Figure 2.8: Whole Tooth Demineralization Sequence in QLF, X-ray and Calcium in Solution for One Sample. Figure shows results from raw data from a single sample (No 5 of ten samples + controls) given as qualitative evidence. The complete data for all assayed samples for X-ray is present in figure 2.4 as quantitative data.

Figure 2.9: (next page) Association between QLF Fluorescence, X-ray Transmittance and loss of calcium in Sequentially Demineralised Bovine Teeth. Decalcification reaches endpoint (equilibrium) in X-ray and QLF assay between 5-10 days and subsequently 5-10 substitutions. Graph shows calcium is no longer released into solution after 13 days, particularly as shown by calcium in solution. Initial drop in QLF fluorescence intensity is representative of the loss in calcium, and therefore related, continued fluorescence in the samples after demineralization (day 8-20) indicates that fluorescence persists and is also independent of the calcium hydroxyapatite.


Mean Transmittance
Mean Autofluorescence
Mean Calcium
Power (Mean Calcium)
Power (Mean Autofluorescence)
Power (Mean Transmittance)
y = 58.351x-0.3576
R2 = 0.9256
y = 39.607x-0.8264
R2 = 0.9267
y = 9.791x-1.5201
R2 = 0.8542
Relative Flourescence/Transmittance Intensity
Calcium in Solution (g/L)
Period of Decalcification (Days)
0
10
20
30
40
50
60
70
0
5
10
15
20
25
0
2
4
6
8
10
12
14
16
18
20
22

2.4.5 Gross Preservation Determination of Towyn-Y-Capel Samples by Autofluorescence against Bovine Demineralization model.

Unlike the model teeth, the ancient teeth as a population showed a greater variety in the size and shape of the samples, predominantly due to the limited sample selection choice. This may have influenced observations compared to modern controls such as those on used to assess the average fluorescence intensity. For example, samples with a smaller surface area gave rise to brighter spectra than would have been expected in a similar or lower net intensity mean compared to a duller larger tooth. Hence, for the Towyn-Y-Capel samples, circular areas enclosing the teeth were used to reduce aberrant background intensity from influencing the overall results. Using an elliptical area, selection circles were drawn around the entire tooth to encompass its surface area but to minimise the background area. From these analyses, information regarding the fluorescence or transmittance intensity were calculated and used for the quantitative analysis and comparison.

It was noted in the early stages of the experiment that fluorescence, as quantified by the QLF system, persisted despite the fact that the mineral faction has been completely removed as indicated by X-ray transmittance analysis and the results calcium loss analysis (Figure 2.8 & Figure 2.9). This result shows that teeth are still able to fluoresce despite complete demineralization, indicating that although the fluorophore responsible for fluorescence is closely associated with the hydroxyapatite matrix and the mineral faction (ie related to the degree of calcification of the sample). The fluorescence itself is independent (although intimately related) to these components of teeth. Previous studies have shown that dityrosine–like compounds may be responsible for this persistence in fluorescence, which has important implications in this research (see Table 2.6).

Dityrosine is a product of Maillard reactions, and it is well established that the oxidation of tyrosine and other amino acids is associated with protein aggregation, cross-linking and fragmentation. Implications of this may be that this fluorophore increases in concentration due to similar oxidation and Maillard reactions, which cause DNA nicking damage and cross-linking. If this is the case, then it is quite possible that despite possible decalcification of ancient samples due to diagenesis that the QLF fluorescence in ancient teeth samples could be relatively higher than the expected levels and in comparison to modern teeth.

Table 2.5: The Variation in the overall QLF Fluorescence of Ancient Teeth					
Tooth Measurement Parameters	**Control Tooth**	**Within the Sampled Towyn-Y-Capel Teeth**			
		Mean[1]	**Std Dev[2]**	**Maximum[3]**	**Minimum[4]**
Mean Fluorescence	98.331	76.853	24.973	133.347	12.514
Standard Deviation	22.887	37.349	9.804	59.694	14.699
Minimum Fluorescence	11	0.8873	0.318	1	0
Maximum Fluorescence	153	158.479	32.035	255	67
Perimeter	1165.531	1607.323	215.452	2093.872	1083.849
Skewness	-0.252	-0.471	0.583	1.649	-1.350
Kurtosis	-0.707	-0.105	1.050	4.827	-1.278

In total analysis was conducted on 71 human ancient teeth. Measurements were performed on the circumference of the enamel fluorescence of the crown area only due to variations in the enamel dentin coverage in different teeth. .Two sample one tail *t*-test assuming unequal variances between modern and ancient teeth, $\alpha=0.05$, 1 degree of freedom, with a hypothesized 20 arbitrary fluorescent units difference. T= 6.314 $\rho=0.079$, which confirms the null hypothesis by 95% [1-4]Mean, Standard Deviation, Maximum and Minimum of the Parameter under investigation (Leftmost column)

Although the optical qualities of mineral loss in teeth has been widely reported, particularly since earlier work in laser fluorescence in the 80's (Bjerlkhagen & Sundstrom, 1981; Bjerlkhagen et al., 1982; Sundstrom et al., 1985), research into the basis of the fluorescence is dental tissues is limited. This is surprising, considering the amount of research, equipment and clinical applications of the technology, is based on this effect. Quantitative Laser Fluorescence (QLF 1), and with the design of more powerful lamps, detections systems and clinical application orientated technology, Quantitative Light Fluorescence (QLF 2).

However, the source of dental tissue fluorescence remains putative and currently identification of the molecules responsible for the effect is more by association with known flurophores than by specific characterization. In addition, there appear to be multiple excitation and emission peaks associated with this fluorescence effect and reports by different authors have suggested that multiple chromaphores may be involved (Table 2.6). For example, it was reported by Hafstrom-Bjorkman (1991) that excitation at 375 nm produced two emission peaks at 460 nm and 560 nm, which was taken as evidence that there were at least two different chromaphores distributed unevenly over the molecular weight faction. These authors proposed that the 460 nm and 560 nm peaks may derive from organic and inorganic components, and that the chromaphore may be incorporated into complexes. This is clearly relevant to the present study as the 560 nm wavelength is that utilised in fluorescence analysis.

Boorij (1982) made some progress in identifying the chromaphores responsible for dental fluorescence by using a variety of analytical techniques. He showed that the fluorescent enamel material in teeth strongly resembled dityrosine and possessed an o-o diphenyl bond. In essence, this work suggested that a complex of cross-linked collagen and hydroxyapaptite bound to the chromaphore was responsible for the fluorescence in teeth and it was speculated that the chromaphore involved had a heterogenous electric dipole structure. In biological tissues, dityrosine is commonly associated with structural proteins, although the chemical nature of this association remains unknown (Matusmoto et al., 1999). As most research is focused on the applications of this technology in clinical uses, particularly as a non-destructive early onset detection technique in dental pathology, it has long been known that the initial decrease in calcium within teeth is reflected by a corresponding decrease in fluorescence.

Clearly, early onset *in-vivo* demineralization research has limited application with regards to ancient teeth. The nature of the chromaphore itself, however, is further highlighted as clearly after the initial correlation between fluorescence and calcium levels, fluorescence levels off to

a background. This is similar to a non-calcium associated intensity level, that may actually correspond to the unbound chromaphore. These results definitely indicate that fluorescence is independent of calcium in the mineralized tissues (Figure 2.8; Figure 2.9).

In relation to the tissues themselves, it can be seen that there is a marked difference in the fluorescence of dentin and enamel in the Towyn-Y-Capel samples that is not reflected in modern samples. This result is all the more pronounced when compared to the enamel fluorescence of ancient samples which is very close to the fluorescence obtained from modern samples, after surface stains and detritus has been removed. This observation indicates that not only has the enamel been highly conserved, but also the associated biological faction (the fluorophore) within the enamel. However, this also shows the poor condition of the dentin in old samples, indicated by the fact that these samples showed poorer fluorescence. For the purposes of this study therefore, only the tooth enamel was used to compare both old and new teeth.

Differences in the fluorescence of dentin may reflect the condition of the substance, and in particular, preservation of the inorganic and organic factions. One explanation for the loss of fluorescence would be the loss of mineral contents in dentin. However, the structure of the dentin in the ancient samples was solid, if not brittle, which is not consistent with some mineral loss, but mainly organic faction, as the elasticity is related to the loss of the collagen component. These observations are consistent with current theories, which suggest that bone and dentin actively associate with the surrounding chemical and physical environment. It is believed that not only do the biological and mineral factions degrade, but also that the latter faction interacts chemically with the local environment, leading to changes in the inorganic and organic composition of dentin (Reference).

In relation to the bovine demineralization model, it can be clearly seen from table 2.5 that the initial level of fluorescence of samples is lower than would be expected in new tissue. Examination of the mean initial fluorescence from the Towyn-Y-Capel population in relation to the percentage fluorescence of the enamel crown area only, reveals that the Towyn-Y-Capel samples exhibit 58% of the mean fluorescence intensity of the control teeth. This result indicates that, on average, the in the Towyn-Y-Capel samples show a 42% net decrease in fluorescence. If the initial fluorescence is linked, as reported, directly to the mineral content or structure of teeth, then this would indicate a loss, or modification of that material to a similar extent.

Table 2.6: Range of Observed Inherent Fluorescent Activity in Dental Tissues.

Observed Excitation λ *	Range of Emission λ *	λ of Specific Fluorescent Peaks*	Associated Source of Fluorescence in Modern Calcified Tissue	References
250-320	425	425	Carious human dentin	Armstrong et al., 1963
250-320	397-402	Not specified	Sound human dentin	Armstrong et al., 1963
280-370	350-520	350,405,450,520	Solid human/bovine animal	Spitzer & Bosch, 1976
280	407	407	Natural dentin	Perry et al., 1969
285	355	355	Fluorophores extracted from dentin	Foreman, 1980
295	360-400	360,400	Hydrolysed enamel & dityrosine	Booij & ten Bosch, 1982
337	400	400	Carious & noncarious enamel	Hafstrom-Bjorkman et al., 1991
337	440-630	440,490,590,630	Carious enamel	Borisova et al., 2006
337	405-525	405,435,490,525	Normal tooth	Subhash et al., 2005
350	410–440	410,440	Not specified	Foreman, 1980
360	410-455	410,455	Organic faction extracted from enamel	Spitzer & Bosch, 1976
365	440-640	440,590,640	Whole collagen & apatite from bone	Bachman & Ellis, 1965
365	430–450	430,450	Natural dentin	Matsumoto et al., 1999
375	460-560	460,560	EDTA dissolved human dental enamel	Hafstrom-Bjorkman et al., 1991
400	480-690	480,624,635,690	Carious enamel	Buchalla, 2005
400	480-687	480,624,650,687	Root carious	Buchalla et al., 2004
405	455-622	455,500,582,622	Normal & carious enamel	Zezell et al., 2006
405	435-555	435,490,555	Dentin level caries	Zezell et al., 2006
405	435-635	435,490,530,635	Pulp level caries	Zezell et al., 2006
405	500	500	Sound & carious enamel	Ribeiro et al., 2005
407	590-700	590,625,635,700	Carious enamel and dentin	Konig et al., 1998
420	495-695	495,595,635,650,695	Dental calculus (supragingival)	Buchalla et al., 2004
460	560	560	Dissolved synthetic hydroxyapatite	Hafstrom-Bjorkman et al, 1991
480-488	540-550	540,550	Carious and noncarious enamel	Hafstrom-Bjorkman et al, 1991, Alfano & Yao, 1981
635	700,783	700,783	Dental calculus (supragingival)	Kurihara et al., 2004
655	720,810	720,810	Carious dentin	Kurihara et al., 2004

Table lists publications and putative sources of endogenous fluorescence, excitation, emission and peak frequencies observed in human teeth. * **λ** – wavelength in ηm Adapted from Bachmann et al., 2006

2.4.6 Histological Preservation of Towyn-Y-Capel Sample

The number and variability inherent in histological animal samples, precludes any statistical approach to their analysis. However, on a subjective basis, the cellular, structural preservation of all the ancient tissues appears to vary from fair to excellent, (HPI 3- HPI 5). This observation is clearly illustrated the samples shown in figures 2.14 to 2.17 as compared to modern samples figures 2.10 to 2.13 and according to the histological preservation index (Figure 2.3).

Although these samples may be representative of the burial environment as a whole, differences have previously been noted that could lead to varying degrees of diagenesis that may show differences across the site. Differences between tissues and their thickness should also be taken into consideration. The elements used for the histological study, namely site associated animal material, generally consisted of less dense bone material than in the human elements, of bone or tooth. Due to the relation of calcified tissue density to structural preservation, it would indicate that the animal bone tissue used for histological studies were likely to degrade faster than the equivalent human tissue, and therefore a good indicator of the relative survival of the structure in human sample.

Finally, some of the fauna found in samples may be post burial additions, or stratigraphic insertions in the archaeological matrix, which could be the underlying reason why the bones are in a very good state of preservation. Overall, the preservation of samples is good for histological preservation from the recovered elements, qualitatively varying between good to excellent as seen from the examples investigated (as shown in Table 2.3).

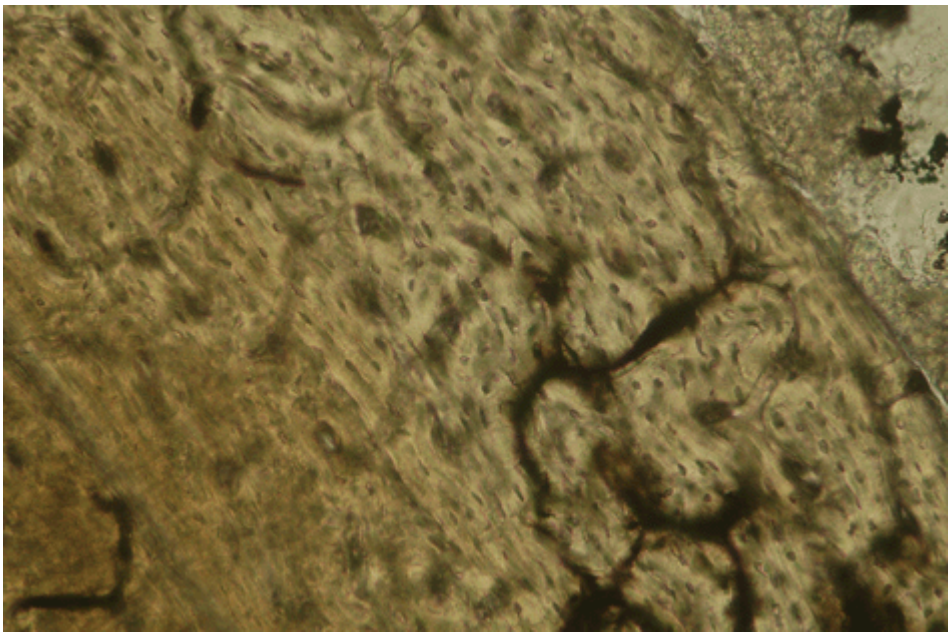


Figure 2.10: Transverse Thin Ground Section (50 µm) of a modern Rabbit Long Bone. Magnification x100 (Histological Photo No 004). Shows surface and centralised cortical structure along with the differences in the Haversian system and Volkmann's canals. Micrograph typical of modern mammalian bone sections observed (5 slide sections and 32 micrographs).

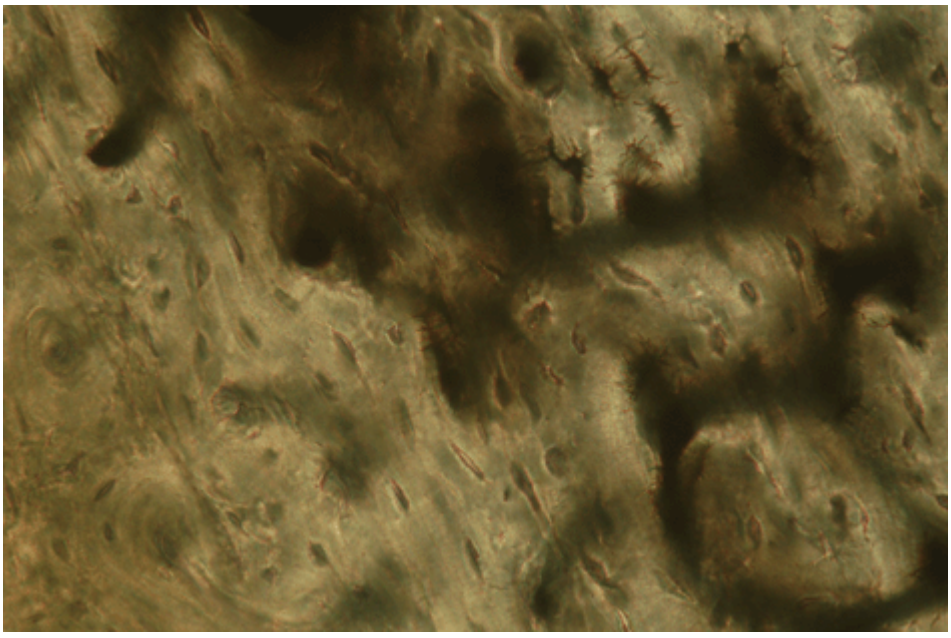


Figure 2.11: Transverse Thin Ground Section (50 µm) of a modern Rabbit Long Bone. Magnification x100 (Histological Photo No 006).Shows lamellar bone Haversian systems interspersed with Volkmann's Canals seen as shadowy areas of out of focus. Micrograph typical of modern mammalian bone sections observed (5 slide sections and 32 micrographs).

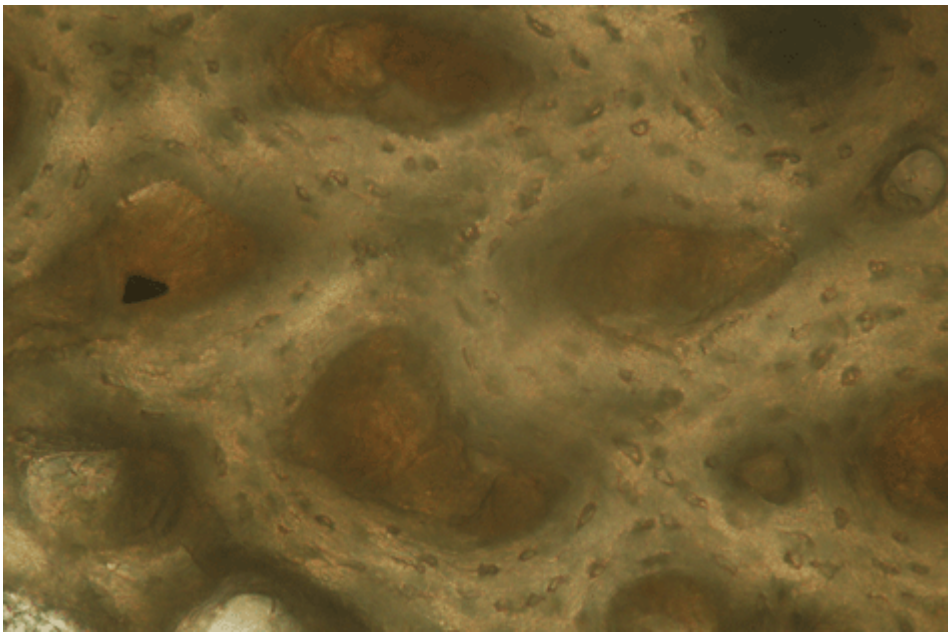


Figure 2.12: Transverse Thin Ground Section (50 µm) of a modern Chicken Long Bone (Diaphysis). Magnification x200 (Histological Photo No 038). High magnification of the Osteon (Havesian system) in modern cortical bone. Micrograph typical of modern avian bone sections observed. (5 slide sections and 38 micrographs).

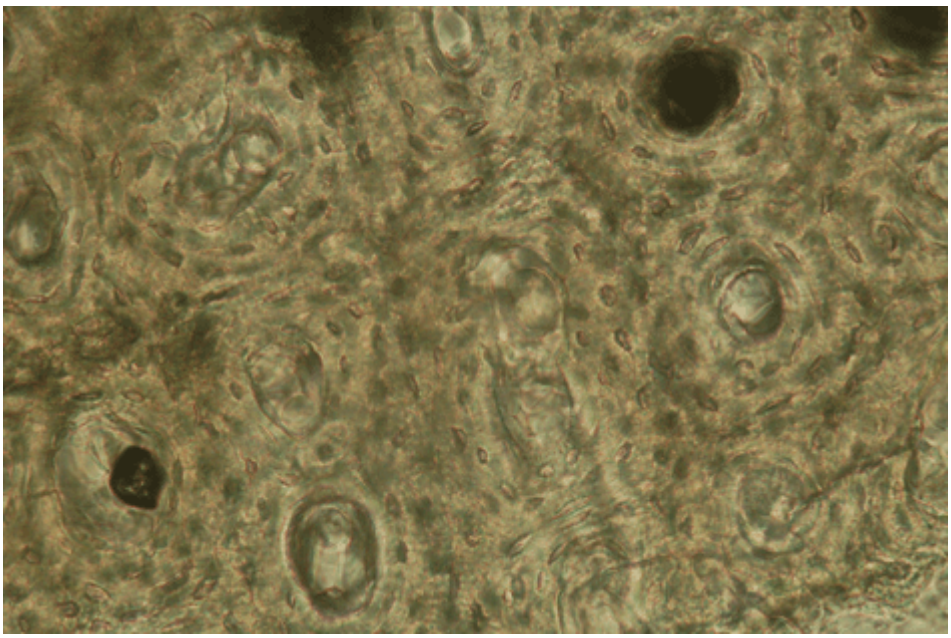


Figure 2.13: Transverse Thin Ground Section (50 µm) of a modern Chicken Long Bone (Diaphysis). Magnification x200 (Histological Photo No 042). High magnification of the Osteon (Havesian system) in modern cortical bone under different light, and in particular the Volkmann's Canal (off centre lower right). Micrograph typical of modern avian bone sections observed. (5 slide sections and 38 micrographs).

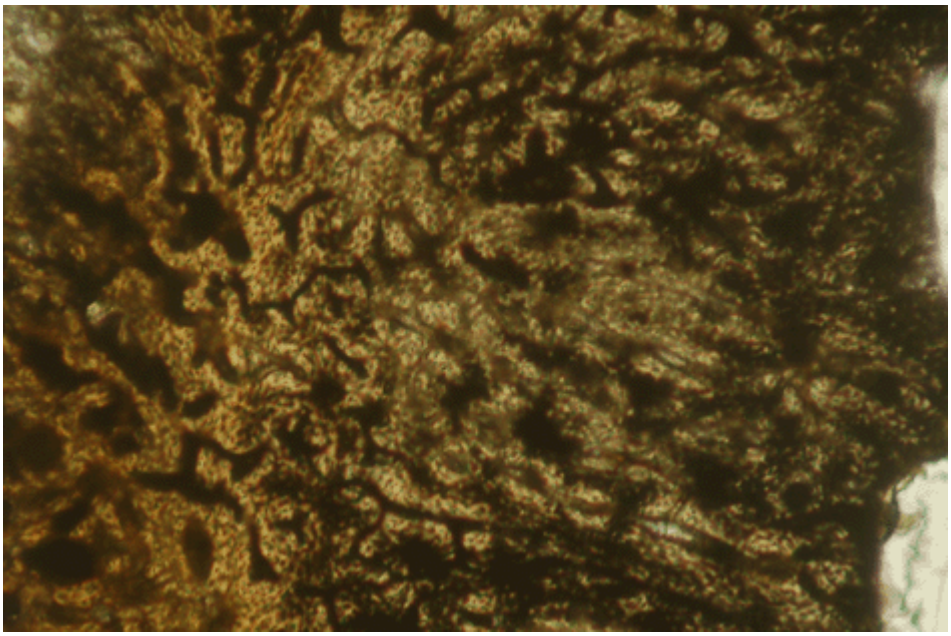


Figure 2.14: Transverse Thin Ground Section (50 µm) of an Ancient Chicken Long Bone (Diaphysis) from Towyn-Y-Capel. Magnification x50 (Histological Photo No 076). Figure shows greater pigmentation than expected under similar conditions for the equivalent modern sample (figure 2.10) which indicates either biological or mineral incorporation into the bone. Micrograph typical of ancient avian bone sections observed. (3 slide sections and 53 micrographs).

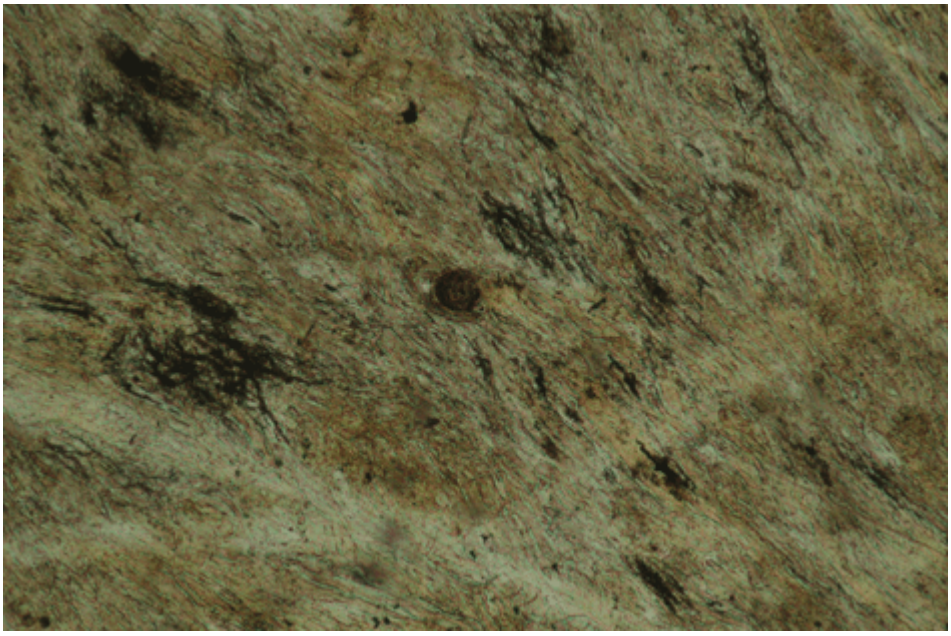


Figure 2.15: Transverse Thin Ground Section (50 µm) of an Unknown Ancient Bone from Town-Y-Capel. Magnification x50 (Histological Photo No 134). Surface focus of sectioned ancient bone material shows fungal hyphae interspersed in the lamellar bone (black hyphal structures) Micrograph typical of some unknown ancient bone sections observed. (6 slide sections and 32 micrographs).

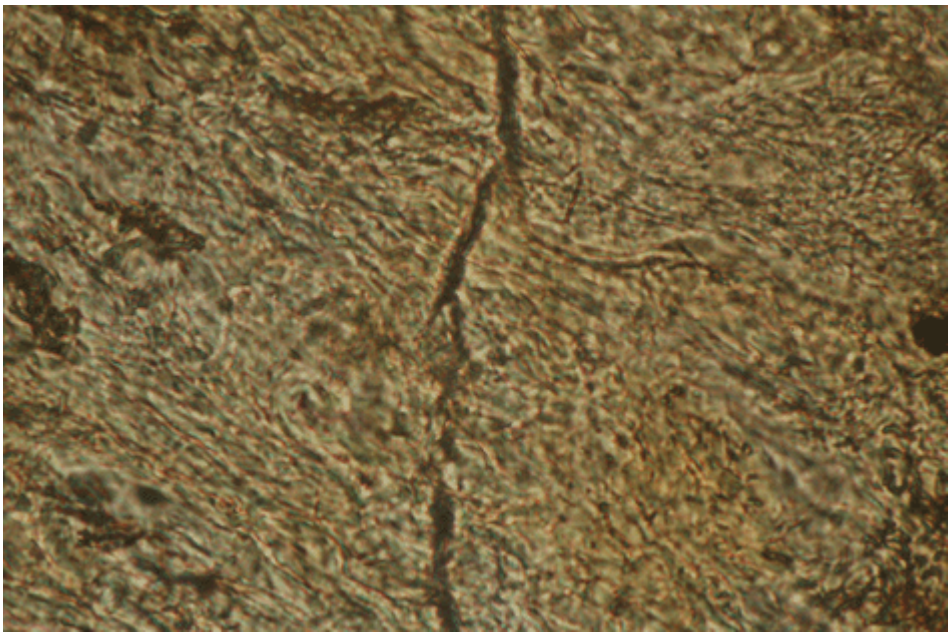


Figure 2.16: Transverse Thin Ground Section (50 µm) of an Unknown Ancient Bone from Towyn-Y-Capel. Magnification x400 (Histological Photo No 140). Figure shows hairline crack caused by compressional damage to the bone structure. Common in ancient material due to diagenetic processes. Micrograph typical of some unknown ancient bone sections observed. (6 slide sections and 32 micrographs).

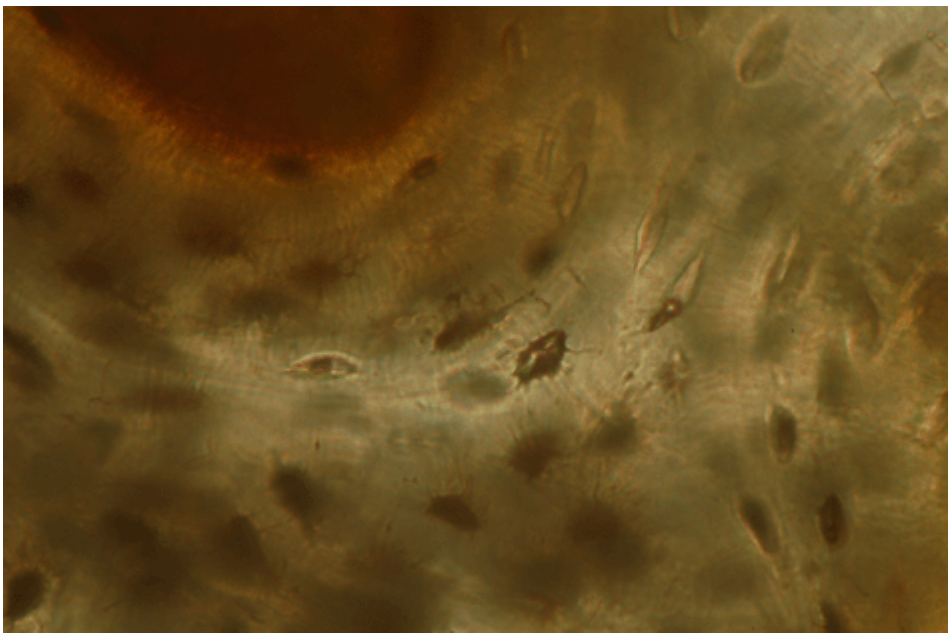


Figure 2.17: Transverse Thin Ground Section (50 µm) of an Unknown Ancient Bone from Towyn-Y-Capel. Magnification x 400 (Histological Photo No 147). High magnification of the Haversian system of ancient bone material from the site shows remarkably good preservation right down to the fine processes of the canaluculi. Micrograph typical of some unknown ancient bone sections observed. (6 slide sections and 32 micrographs).

Table 2.7: Histological Photo Numbers and Details for Modern Control Samples

No	S#	MA	HPI	Source	Bone	No	S#	MA	HPI	Source	Bone
1	D	50	N/A	Rabbit	LB	36	I	200	N/A	Chicken	LB.D
2	D	50	N/A	Rabbit	LB	37	I	200	N/A	Chicken	LB.D
3	D	100	N/A	Rabbit	LB	38	I	200	N/A	Chicken	LB.D
4	D	100	N/A	Rabbit	LB	39	I	200	N/A	Chicken	LB.D
5	D	200	N/A	Rabbit	LB	40	J	50	N/A	Chicken	LB.D
6	D	200	N/A	Rabbit	LB	41	J	100	N/A	Chicken	LB.D
7	D	200	N/A	Rabbit	LB	42	J	200	N/A	Chicken	LB.D
8	D	400	N/A	Rabbit	LB	43	J	200	N/A	Chicken	LB.D
9	D	400	N/A	Rabbit	LB	44	J	400	N/A	Chicken	LB.D
10	D	400	N/A	Rabbit	LB	45	J	400	N/A	Chicken	LB.D
11	D	1000	N/A	Rabbit	LB	46	K	50	N/A	Chicken	LB.D
12	D	1000	N/A	Rabbit	LB	47	K	50	N/A	Chicken	LB.D
13	D	40?	N/A	Rabbit	LB	48	K	50	N/A	Chicken	LB.D
14	E	50	N/A	Rabbit	LB.E	49	K	100	N/A	Chicken	LB.D
15	E	100	N/A	Rabbit	LB.E	50	K	200	N/A	Chicken	LB.D
16	E	200	N/A	Rabbit	LB.E	51	K	200	N/A	Chicken	LB.D
17	E	400	N/A	Rabbit	LB.E	52	K	400	N/A	Chicken	LB.D
18	F	50	N/A	Rabbit	LB.E	53	K	400	N/A	Chicken	LB.D
19	F	100	N/A	Rabbit	LB.E	54	K	1000	N/A	Chicken	LB.D
20	F	200	N/A	Rabbit	LB.E	55	K	1000	N/A	Chicken	LB.D
21	F	200	N/A	Rabbit	LB.E	56	L	50	N/A	Chicken	LB.D
22	F	400	N/A	Rabbit	LB.E	57	L	100	N/A	Chicken	LB.D
23	F	100	N/A	Rabbit	LB.E	58	L	200	N/A	Chicken	LB.D
24	G	200	N/A	Rabbit	LB.E	59	L	400	N/A	Chicken	LB.D
25	G	200	N/A	Rabbit	LB.E	60	L	400	N/A	Chicken	LB.D
26	G	200	N/A	Rabbit	LB.E	61	L	1000	N/A	Chicken	LB.D
27	G	200	N/A	Rabbit	LB.E	62	M	50	N/A	Chicken	LB.D
28	H	50	N/A	Rabbit	N/A	63	M	100	N/A	Chicken	LB.D
29	H	50	N/A	Rabbit	N/A	64	M	100	N/A	Chicken	LB.D
30	H	100	N/A	Rabbit	N/A	65	M	200	N/A	Chicken	LB.D
31	H	200	N/A	Rabbit	N/A	66	M	200	N/A	Chicken	LB.D
32	H	200	N/A	Rabbit	N/A	67	M	400	N/A	Chicken	LB.D
33	I	50	N/A	Chicken	LB.D	68	M	400	N/A	Chicken	LB.D
34	I	100	N/A	Chicken	LB.D	69	M	400	N/A	Chicken	LB.D
35	I	100	N/A	Chicken	LB.D	70	M	400	N/A	Chicken	LB.D

Table lists Micrographs taken from multiple observations of modern bone sections. Slide numbers and magnification and photo numbers recorded for comparison and analysis. Rabbit- *Oryctolagus cuniculus,* Chicken - *Gallus gallus domesticus* , LB-L*ong Bone,* E-*Epiphysis,* D-*Diaphysis,* MA-*Angular Magnification* , HPI-*Histological Preservation Index.* S#- *Slide Number*. Slide Numbers correspond to transverse sections as outlined in Table 2.3. HPI does not apply as all samples are modern and given a default of Excellent Preservation -5. For more details see Figure 2.3. Photo Numbers correspond to digital photo in Appendix 2.3.

Table 2.8: Histological Preservation Index, Photo Numbers &Details for Ancient Specimens

No	S#	MA	HPI	Source	Bone	No	S#	MA	HPI	Source	Bone
71	N	50	5	Uk	Uk	119	Q	100	4	Chicken	LB.D
72	N	100	5	Uk	Uk	120	Q	100	5	Chicken	LB.D
73	N	200	5	Uk	Uk	121	Q	200	5	Chicken	LB.D
74	N	1000	5	Uk	Uk	122	Q	200	5	Chicken	LB.D
75	O	50	4	Chicken	LB.D	123	Q	200	5	Chicken	LB.D
76	O	50	4	Chicken	LB.D	124	Q	200	n/a	Chicken	LB.D
77	O	50	4	Chicken	LB.D	125	Q	400	5	Chicken	LB.D
78	O	50	4	Chicken	LB.D	126	Q	400	5	Chicken	LB.D
79	O	50	4	Chicken	LB.D	127	Q	400	5	Chicken	LB.D
80	O	50	4	Chicken	LB.D	128	R	100	n/a	Uk	Uk
81	O	100	4	Chicken	LB.D	129	R	100	n/a	Uk	Uk
82	O	100	5	Chicken	LB.D	130	R	200	n/a	Uk	Uk
83	O	100	5	Chicken	LB.D	131	R	200	n/a	Uk	Uk
84	O	100	5	Chicken	LB.D	132	R	200	n/a	Uk	Uk
85	O	100	5	Chicken	LB.D	133	R	200	n/a	Uk	Uk
86	O	100	3	Chicken	LB.D	134	S	50	3	Uk	Uk
87	O	100	4	Chicken	LB.D	135	S	50	3	Uk	Uk
88	O	200	3	Chicken	LB.D	136	S	100	3	Uk	Uk
89	O	200	5	Chicken	LB.D	137	S	100	3	Uk	Uk
90	O	200	4	Chicken	LB.D	138	S	200	3	Uk	Uk
91	O	200	4	Chicken	LB.D	139	S	200	3	Uk	Uk
92	O	200	4	Chicken	LB.D	140	S	400	3	Uk	Uk
93	O	400	3	Chicken	LB.D	141	S	400	3	Uk	Uk
94	O	400	4	Chicken	LB.D	142	T	50	2	Uk	Uk
95	O	400	4	Chicken	LB.D	143	T	50	2	Uk	Uk
96	O	400	5	Chicken	LB.D	144	T	100	3	Uk	Uk
97	O	1000	4	Chicken	LB.D	145	T	100	3	Uk	Uk
98	P	50	4	Chicken	LB.D	146	T	200	4	Uk	Uk
99	P	50	3	Chicken	LB.D	147	T	400	4	Uk	Uk
100	P	50	4	Chicken	LB.D	148	V	50	2	Pig/Sheep	L.B.D
101	P	100	4	Chicken	LB.D	149	V	50	2	Pig/Sheep	L.B.D
102	P	100	4	Chicken	LB.D	150	V	100	3	Pig/Sheep	L.B.D
103	P	100	3	Chicken	LB.D	151	V	100	3	Pig/Sheep	L.B.D
104	P	200	5	Chicken	LB.D	152	V	100	3	Pig/Sheep	L.B.D
105	P	200	4	Chicken	LB.D	153	V	100	3	Pig/Sheep	L.B.D
106	P	200	4	Chicken	LB.D	154	V	200	3	Pig/Sheep	L.B.D
107	P	200	4	Chicken	LB.D	155	V	200	3	Pig/Sheep	L.B.D
108	P	400	4	Chicken	LB.D	156	V	400	3	Pig/Sheep	L.B.D
109	P	400	4	Chicken	LB.D	157	V	400	3	Pig/Sheep	L.B.D
110	P	400	4	Chicken	LB.D	158	W	50	3	Uk	Uk
111	P	400	4	Chicken	LB.D	159	W	50	4	Uk	Uk
112	P	400	n/a	Chicken	LB.D	160	W	100	5	Uk	Uk
113	P	1000	n/a	Chicken	LB.D	161	W	100	5	Uk	Uk
114	P	1000	n/a	Chicken	LB.D	162	W	200	5	Uk	Uk
115	Q	50	4	Chicken	LB.D	163	W	200	5	Uk	Uk
116	Q	50	3	Chicken	LB.D	164	W	400	5	Uk	Uk
117	Q	50	4	Chicken	LB.D	165	W	400	5	Uk	Uk
118	Q	100	4	Chicken	LB.D						

Table lists Micrographs taken from multiple observations of ancient bone sections and qualitative HPI score.. Slide numbers and magnification and photo numbers recorded for comparison and analysis. See Table 2.7 for species and magnification details. HPI-*Histological Preservation Inde.*. Slide Numbers correspond to transverse sections as outlined in Table 2.3. HPI determined as outlined in Figure 2.3 conditions vary from 1-Poor to 5 – Excellent. Photo Numbers correspond to digital photo in Appendix 2.3

2.5 Final Discussion and Conclusions

Osteological information is clearly imperative for the appropriate selection of samples for further biochemical consideration, and it is important to consider the gross morphology of the individual skeletal element, compared not only to previous populations and studies but within the burial population itself. The additional benefit of gross morphology and appropriate recording for later destructive analysis is the ability to revisit the analysis at a latter date without losing valuable information.

The ability to supplement this technique with an equally non-invasive and simple technique such as QLF is potentially important. With this new approach we could ascertain further information regarding the tissues chemical status (concentration of calcium at the surface), without invasive or destructive sampling. This is very important and worth continuing. The weakness with the technique as it stands is the absence of a validated reference or calibration curve for whole tooth analysis as was required for this study. With further experimentation and validation this technology has the capabilities of being a great screening technique. As to the fluorescence itself their is a “bare-bones” understanding that the lack or presence of fluorescence is not a direct indicator of the inorganic status of a bone or tooth, but an estimate, as fluorescence exists within the sample despite the absence of calcium, just at a lower level. Clearly an important area to pursue with regards to this research, although outside of the stipulation of this thesis, is the specific nature of the fluorescent molecule itself and what affect that diagenetic processes may have on the molecule, and how exactly it is incorporated into the hydroxyapatitic structure of bones and teeth (see references Table 2.6).

With regards to the histological assessment of preservation, although relevant to the assessment of biological condition, in this study it did not bring to light any more information regarding the state of the samples that had not already been ascertained by gross morphology. In addition, the procedure involved a great deal of destructive sampling, which is why ancient animal bones were used in place of the limited human material. It must also be borne in mind that the sectioning and grinding technique themselves are not immune to the introduction of aberrant damage itself that, if left unchecked can introduce artificial artefacts into the results (Maat et al., 2001).

Regardless the high degree of histological preservation of the ancient animal samples does indicate that subsequent chemical and biological investigations have a greater chance of success.

CHAPTER 3

CHEMICAL ANALYSIS OF THE INORGANIC AND ORGANIC FACTIONS OF SELECTED TOWYN-Y-CAPEL SKELETONS

3 CHEMICAL ANALYSIS OF THE INORGANIC AND ORGANIC FACTIONS OF SELECTED TOWYN-Y-CAPEL SKELETONS

3.1 Introduction

In order to maximise retrieval of forensic information from the Towyn-Y-Capel population, a range of genetic, biochemical and physical techniques were employed in this study. As previously discussed (see §1.3.3, §1.3.4 & §1.3.6) investigations using non-genetic molecular techniques such as High Performance Liquid Chromatography (HPLC) and Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) can provide important data regarding the state and degree of preservation of hard ancient tissue samples. These data have the potential to complement the results obtained from the use of genetic molecular techniques such as DNA amplification and also to provide additional information, particularly with regard to the diet and residence patterns of the Towyn-Y-Capel population. ICP-MS analysis was undertaken by myself and colleagues at the Liverpool Dental School (University of Liverpool) and amino acid D/L enantomer specific analysis performed by Microanalytica LLC (Miami, Florida, USA).

3.2 Materials and Methods

It was found that the archaeological skeletal population showed a wide variation in condition, which precluded the full anthropological analysis of this population in its entirety. As a result, this investigation was limited to a small representative sample of the skeletal population, comprising approximately 8% of the total recovered intact skeletal assemblages. Morphological, histological and genetic analyses of patella and dental samples in previous chapters have demonstrated that the dental samples were in far better condition than the former. Given the well established advantages of dental enamel as a diagenetically resistant material, it was decided to use teeth as the primary samples for investigation in this chapter.

Accordingly, the same set of 10 samples (Table 3.1) that were chosen for mtDNA sequencing (see §5) on the basis of skeletal integrity, condition, preservation, dental availability and, where possible, archaeological context, in selecting representative samples that were distributed throughout the archaeological site.

The varying condition of both skeletons and the archaeological site coupled to the limited availability of archaeological material, made uniform sample selection difficult. In practice, therefore, many teeth were selected primarily on the basis of the quantity of material (tooth size), and the integrity of the enamel cusp and the tooth (see §1.2.4; §2.3.2). To maximise reproducibility, duplicate teeth of similar dental position and preservation were selected with an order of priority that was: third, second and then first molars, with mandibular molars chosen over maxillary molars.

Any teeth that were deemed to be non-intact were recorded, which was particularly important as these teeth are more susceptible to possible sources of contamination. The human control samples consisted of three deciduous teeth from young individuals who resided on Holy Island, Anglesey, (kindly provided by R. Smith) and a horse tooth, which was recovered from the archaeological site and served as a site control). The soil control was selected as that found at the interface layer of humic soil material wherein the majority of skeletons were interred (§1.1.2 Course sand between contexts 159 & 189).

Selected dental samples were subjected to surface abrasion and decontamination techniques as described in chapters 2 & 4 (§2.2.1, §4.1). All procedures were performed under sterile conditions and, where possible, with periodic UV decontamination. For each tooth under analysis, a customized thin-wire dental band saw and sterile tweezers were used to remove two small segments of the side cusp of the dental enamel. These teeth segments were then retained for ICP-MS experiments. The remaining tooth materials were then further irradiated using UV light and kept for sample preparation as discussed in chapter 5 (§ 5.1).

Table 3.1: Anthropological and Taphonomical Analysis of the 10 Skeletal Samples from Towyn-Y-Capel Used In Chapter 3

Sk	Tooth[1]	Cond[n2]	Age[3]	Sex[2]	GHI[4]	EFI [5]	Skeletal Pathology/ Comments[2]
Sk21	RM^3	Mod	Adult	M	2	25.16	None
Sk33	RM_3	Mod	20-30	F	2	38.74	Osteoblastic lesion on R occipital/parietal
Sk34	LM_3	Mod	35-45	M	2.6	56.00	Abscess left maxilla PM1-M2
Sk35	LM_2	Good	15-25	M	1	42.63	Slipped epiphysis/ pseudoarthrosis R hip, Schmorl's nodes, scoliosis
Sk41	RM_2	Mod	30-45	M	2	45.19	Osteitis L tibia/fibula
Sk51	LM_3	Mod	30-49	M	3	46.34	Cribra orbitalia, unfused sacrum s1-s2, osteophytes T10, periostitis anterior tibia, mandible abscess, subchondral cyst distal end prox phalanx 1 (R foot)
Sk52	RM_2	Poor	25-45	F?	2.4	47.93	Cervical osteophytes-arthritic, dental overcrowding, metopic suture
Sk56	LM_3	Good	50-60	M	N/A	51.25	Sinusitis, dental anomalies
Sk57	N/A	Mod	25-35	?	N/A	59.10	Additional lumbar vertebra, rotated mandibular premolar, extensive periodontal disease
Sk60	LM_1	Good	35-45	F	N/A	66.36	Cribra orbitalia, thoracic osteophytes, midshaft healed parry-type fracture L ulna, Larm disused? Hammer deformity of 5th R toe , fusion mid-dist phalanges

The table above outlines the skeleton and tooth details of the samples used for the following amino acid composition/racemization, trace element concentration and Strontium Isotope ratio determination and analysis. [1]Tooth position and type used for the analysis, §1.2.4 for more detail regarding nomenclature; [2]Estimates utilizing qualitative data from corresponding osteological investigation. [3]Age given according to anthropological evidence, determined in years unless stated otherwise; [4]Gross Histological Index (GHI) of the dental sample itself, as determined previously (§2.3.2) and range from 1 -4 (1-Poor to 4-Excellent); [5]Estimated Fluorescence Integrity (EFI) as determined previously (§2.3.5), percentage integrity as determined by standardized fluorescence intensity assay.

3.2.1 Determination of Amino Acid Concentration and D/L Racemization in Selected Enamel Samples by using Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Selected dental samples were cleaned and reduced to dental powder under nitrogen using the protocol described in chapter 4 (§4.3). Under aseptic conditions, aliquots of between 15 and 50 mg of this dental powder (Table 3.2) were then weighed into vials as were modern tooth samples, which were donated by a local dentist, and which served as controls. All samples were then sent to Microanalytica LLC for determination of their amino acid content, and analysis by D/L enantomer specific reverse phase high performance liquid chromatography, according to established methodology (Klinken & Mook, 1990).

3.2.2 Determination of Trace Element and Strontium Isotope Concentrations in Selected Enamel Samples by Multicoupler Inductively Coupled Plasma Mass Spectrometry (MC ICP-MS)

The outer surface of each tooth was repeatedly rinsed with sterile ddH_2O during mechanical cleansing. This procedure was used to eliminate diagenetic material, detergent wash contamination, remnants of dentin and enamel post-sectioning, and other surface contaminants, which were manifested as a discoloration of the teeth. Enamel sections were sonicated, and dissolved in ultrapure nitric acid (0.1% v/v). Soil samples were treated overnight with ultrapure nitric acid (1% v/v) according to Hoppe *et al*., (2003).

Solubilised teeth were analysed for trace elements Magnesium(^{24}Mg), Manganese (^{55}Mn), Iron (^{56}Fe), Copper (^{65}Cu), Zinc (^{66}Zn), Strontium (^{88}Sr), Cadmium (^{114}Cd), Tin (^{124}Sn) and Lead (^{208}Pb) (Method 1) followed by assay for ^{84}Sr, ^{86}Sr, ^{87}Sr & ^{88}Sr isotopes of strontium (Method 2). Both assays were performed using a MultiCollector Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS)(VG PQExCell TermoElemental) at the University of Liverpool. The values were expressed as µg/g of tissue or ppm, the latter being convention in ICP-MS analysis and as such was used the following sections,

Trace Element Quantification

Reagents

Standard stock for all reagents was VWR (International LLC) ICPMS Grade. Internal standard (I/S) diluent contained 50 ppb indium and 50 ppb gallium in 0.1% Nitric acid, made from VWR stock reagents (In Ga 1000 ppm ICPMS standard grade) Ultrapure Nitric acid (Merck) and 18 MΩ water.

The solvent used for all reagents was VWR ICPMS Grade. Internal standard diluent (I/S) contained indium (50 ppb) and gallium (50 ppb) in nitric acid (0.1% v/v). This solvent was prepared using ICPMS standard grade indium and gallium (VWR), ultrapure nitric acid (Merck) and water with a conductivity of 18 MΩ

Instrument Protocol

The collision gas used in these experiments was 2% hydrogen in helium (B.O.C.). Samples were diluted by adding 150 µ of material to 4.0 ml of I/S diluents. Samples were regularly agitated to ensure homogenous mixing and experiments were performed in duplicate. The Instrument (VG PQExCell TermoElemental with a Cetac ASX500 Autosampler) was optimised for the flow rates of argon and the collision gas rate was 3 *15 second samplings per dilution repeated in triplicate.

Strontium Isotope Quantitiation

Reagents

Internal standard diluent made up of 10 ppb indium, 10 ppb gallium in 0.1% Nitric Acid (VWR stock reagents, Ga 1000 ppb (ICPMS standard grade), Ultrapure Nitric acid (Merck) and 18 MOhm water. Standards were NIST SRM 987, and Blank 0.1% Nitric acid. Intermediate standard solution made from solid dissolved in 0.1% ultapure Nitric acid to give approximately 200 ppm in solution. The working solution was made up the intermediate solution diluted with the internal standard diluent to give approximately 20 ppb.

Instrument Protocol

The Instrument (VG PQExCell TermoElemental with a Cetac ASX500 Autosampler) was optimised for argon gas flow rates, at a rate of 1* 30 second sampling per dilution. The samples were analysed according to the protocol: “Blank - SRM 987 - Blank - Unknown solution 1- Blank - SRM 987 - Blank - SRM 987 - Blank - Unknown solution 2 - Blank - SRM 987 - Blank - Unknown solution 3- Blank - SRM 987.....SRM 987” in order that each strontium containing solution was preceded by a blank and each unknown was bracketed by a pair of SRM 987 dilutions. Dilution samples were diluted according to the assayed strontium value in I/S diluent to give a concentration of approximately 20 ppb to ensure a similar detector response for all solutions and repeated in triplicate

ICP-MS Calculations 2

Each solution was "blank corrected" by subtracting the counts obtained from the preceding blank for the appropriate isotope, and the measured isotopic ratios were then calculated. For each individual sample a K factor (correction factor obtained from the measured and actual isotopic ratios of the SRM) was obtained for the mean values of the bracketing SRM dilutions. This factor was then applied to the samples measured ratio values. All values were expressed as ppm or ppb of the original tooth sample.

3.3 Results and Discussion

3.3.1 Determination of Amino Acid Composition and D/L Racemization in Towyn –Y-Capel Samples

The relative composition of aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glycine (Gly) and alanine (Ala), which are the predominant amino acids of collagen, were determined. The results of these analyses are shown in figure 3.1.

Modern calcified tissues contain collagen in a consistent proportion. In collagen, the percentage content of each amino acid, compared to the total amino acid contents in teeth for Asp, Ala, Ser and Glu are 5.1%, 11.3%, 3.5% and 7.5%, respectively (Klinken & Mook, 1990). According to Klinken & Mook (1990), a standard "degree of preservation and purity" can be established by comparing the relative proportions of amino acid residues in collagen to a standard (modern bone). In addition to establishing the "preservation" of a sample, this parameter provides a quantitative assessment of the absolute concentration of amino acids present in samples. Essentially, this establishes a correlation between the composition of amino acids in a sample and its state of biological preservation.

The values shown for the 'degree of preservation and purity' of skeletal samples in figure 3.1 appear to be relatively uniform compared to the control, with levels between 40-75% of the expected "ideal" value. This observation indicates some degradation but does not indicate exogenous amino acid contamination or protein sources other than collagen. It can be seen from figure 3.1 that skeleton samples Sk34, Sk52, Sk59 and Sk60 show the best states of preservation, exhibiting over 60% of the amino acid levels of a modern tissue sample. Interestingly, the control tooth sample, although recently extracted, shows a particularly low amino acid composition, compared to the archaeological samples. It is possible that this observation results from post extraction procedures, which involved transport in a bleach solution, and such solutions has been shown to cause damage to tooth structure, and in particular, the organic faction.

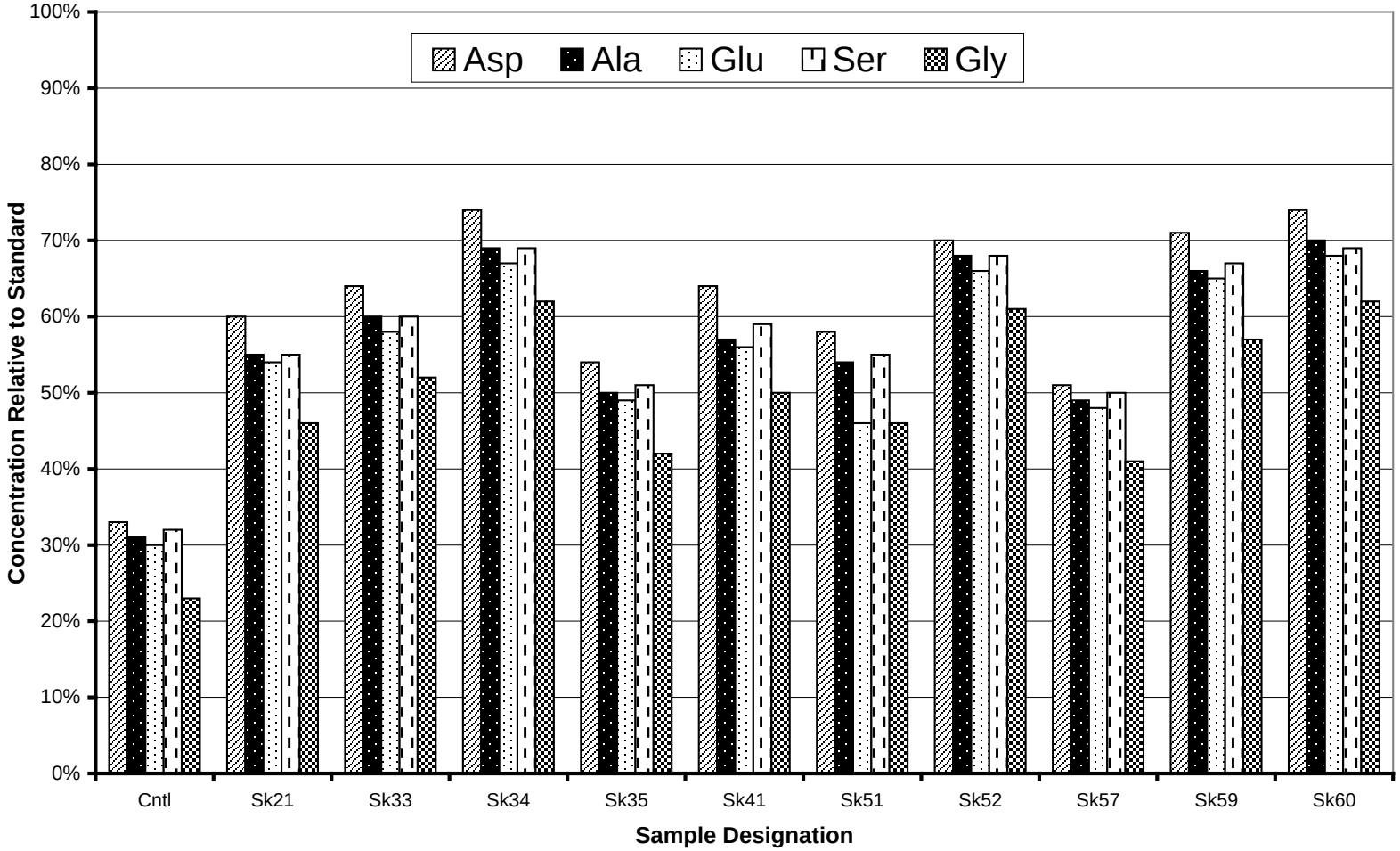


Figure 3.1: Collagen Specific Amino Acid Composition of Towyn-Y-Capel Samples. Bar charts shows the relative concentrations of Aspartate (Asp), Alanine (Ala), Glutamine (Glu), Serine (Ser) and Glycine (Gly) to a standard reference tooth. Samples measured include the bleached control tooth and teeth from Skeletons 21, 33, 35, 41, 51, 52, 57, 59 and 60 (as shown in Table 3.1).

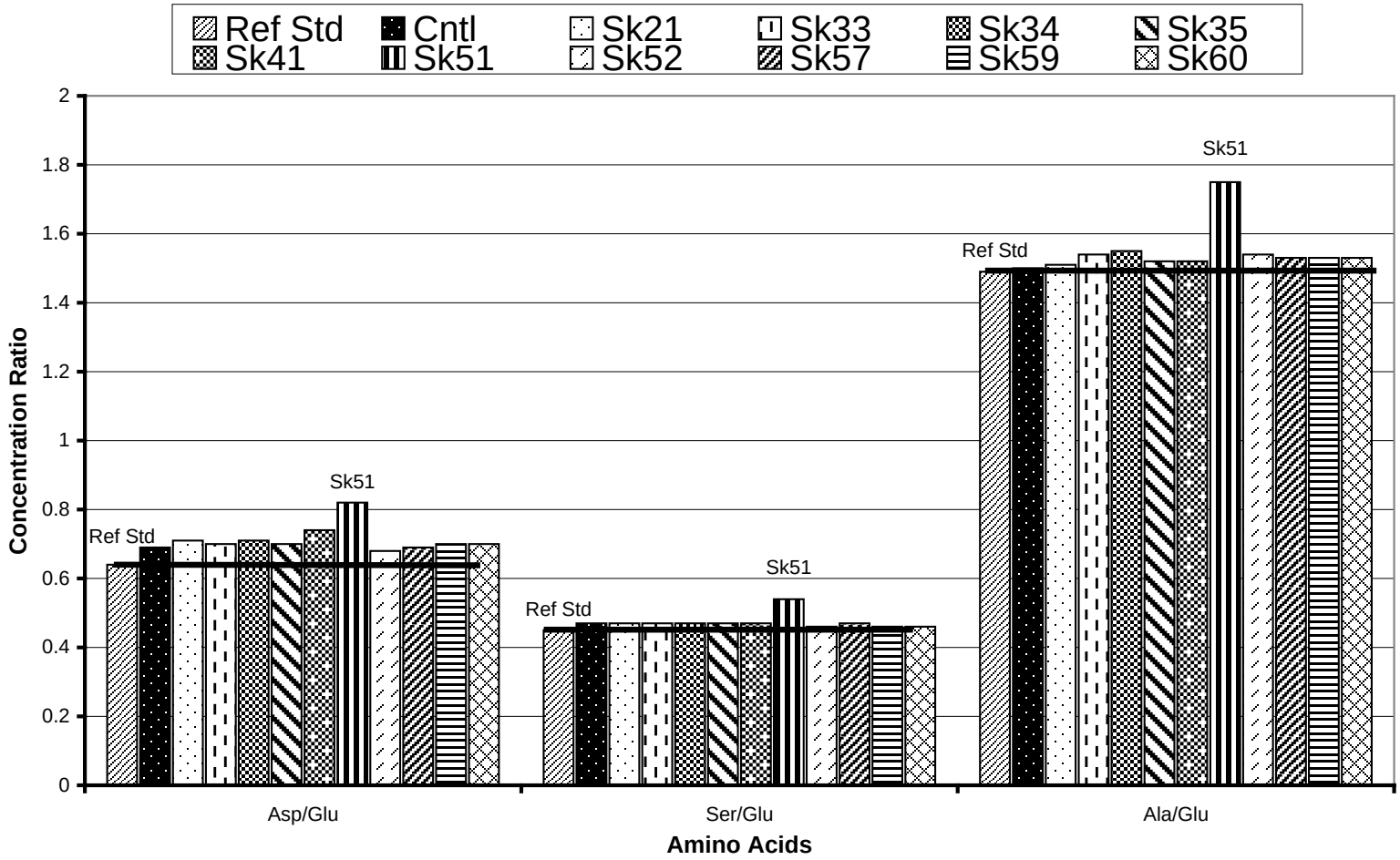


Figure 3.2: Comparison of Amino Acid Composition using Glutamic Acid Ratio. Bar charts showing concentration ratios between Aspartate (Asp), Serine (Ser) and Alanine (Ala) against Glutamine (Glu). Horizontal black line indicates expected ratio from the standard Reference compared to the bleached control tooth and teeth from Skeletons 21, 33, 35, 41, 51, 52, 57, 59 and 60 (as shown in Table 3.1). Sk51 shows elevated concentration ratios compared to the population and the standard.

Determination of Amino Acid Composition Ratio of Medieval Samples

The data in figure 3.2 shows a comparison of the relative levels of the amino acids (Asp, Ser, Ala, Glu) within each skeletal sample to a reference standard (modern bone), and they are shown as a line extrapolated across the ratios of the other samples. These histograms indicate the degree of preservation of each amino acid within a sample as a function of a modern sample. The specimen with the highest peak within each comparison indicates that which is best preserved. Surprisingly, all the analysed samples have a higher ratio than the control. However, this observation probably results from the bone used as a control, which may have a slightly different collagen level compared to teeth. The ratios for all samples therefore appears to be within limits, or proximal to the expected ratios for ancient but well preserved samples. These ratios are therefore good indicators of the state of preservation of the organic faction of the teeth investigated and hence for selecting candidates for successful aDNA study. The high peak of the sample from Sk51, however, appears anomalous across all three amino acid ratios. This indicates that, per unit mass, Sk52 has a higher level of the amino acids than the modern control, which is inconsistent with the age of the sample and the higher collagen content of bone as compared to teeth. It may be that this anomalous result was due to the presence of contaminating organics and so this sample was excluded from further investigations.

Determination of Aspartic Acid Racemization of Medieval Samples

As discussed previously (see §1.3.6), aspartic acid is the first among the amino acids to racemize, and since its rate of degradation is similar to that of DNA (Bada et al., 1984). Therfore, it is particularly useful as an indicator for biomolecular research using ancient samples. The D to L ratio of Asp (Asp D/L) of skeletal samples is given in figure 3.3. The degree of racemization and hence level of degradation of the biological faction of the calcified tissues can be clearly seen, particularly when viewed against the bone standard (Ref Std). With most samples showing D/L ratios around 0.08, which is a value *circa* 2.5 times greater than those of the modern bone standard, the aspartic acid in these samples has undergone a significant D/L transformation. As archaeological material, some sample racemization is to be expected and it is generally agreed that for successful DNA retrieval, a cut off value for Asp D/L of around 0.1 is the acceptable limit of racemization. This is, however, a ‘Rule of thumb’, and according to the research published by Poinar (1996) and Bada (1994) in which DNA in values of around 0.08 and above represent samples that are extremely fragmented. Moreover, the efficient and reproducible recovery of

genetic material from these samples is unlikely. However, Poinar (1999) reported the successful recovery of genetic material from an ancient specimen with a D/L ratio of 0.12.

Of note, is the observation that most samples, in particular Sk21, Sk34, Sk41, Sk59 and Sk60, have an Asp D/L ratio that is equal to, or higher than, 0.08. In the cases of Sk51 and Sk52, Asp D/L ratios are well in excess of 0.07. For the remaining samples, the lowest degree of racemization, is between 0.066 - 0.070, and is from skeletal samples Sk33, Sk35 and Sk57. These latter samples, therefore, demonstrated the best states of preservation and the highest potential for the recovery of genetic material. Unexpectedly, the degree of racemization shown in the modern tooth control was very high, which, along with the specific amino acid compositions, demonstrated that the biological faction of the modern tooth sample was in a particularly poor state. This may have been due to prolonged bleach treatment of the teeth prior to laboratory examination.

Determination of Comparative Alanine Racemization in Medieval Samples

The racemization of Ala (Ala D/L) is a considerably slower process than that of Asp (§1.3.6), and hence D/L racemization ratios should be less than those of Asp for a given sample. If, in a given sample, Ala D/L> Asp D/L, then this could indicate the possibility of contamination. The values obtained for Ala racemization of between 0.008 and 0.011 are consistent with the presence of endogenous amino acids, and consequently are taken to exclude the possibility of exogenous or contaminant amino acids. The Ala D/L ratios of Sk35, Sk52, Sk57 and Sk59, however, are lower than those of the reference standard and control. This result may be a reflection of the lack of sensitivity at very low levels of D/L racemization, or the different conditions within bone compared to teeth (i.e. the difference of the standard reference and the skeletal samples). However, this is difficult to determine as the results for the modern tooth control were anomalous as shown in figures 3.1 and 3.3, due to low overall amino acid levels, and high aspartic acid racemization (as ultimately racemization is a factor of collagen preservation and not sample age or taphonomical condition). Alternatively, the four samples may have received some low level biological contamination that has not shown in the previous amino acid composition profiles and comparative ratio analysis.

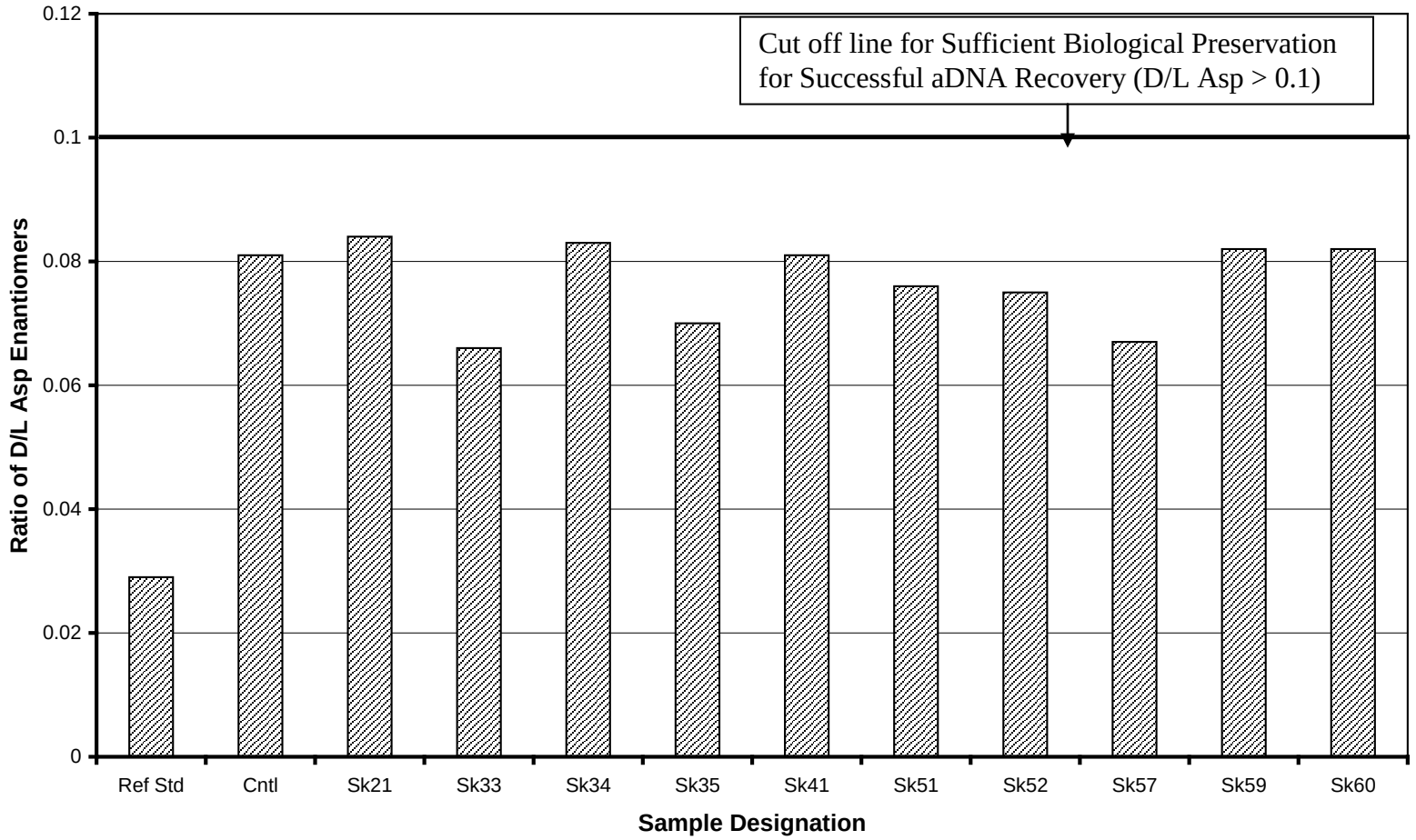


Figure 3.3: Aspartic Acid Racemization in Towyn-Y-Capel Population Samples. Barchart compares the D/L ratio of Asp in the sampled skeleton teeth (Table 3.1), bleached tooth control and reference standard. Horizontal line at 0.100 Asp D/L represents the limit of racemization with regards to aDNA recovery and protein analysis. Sk33, 35 and 57 show the lowest ratios amongst the samples .

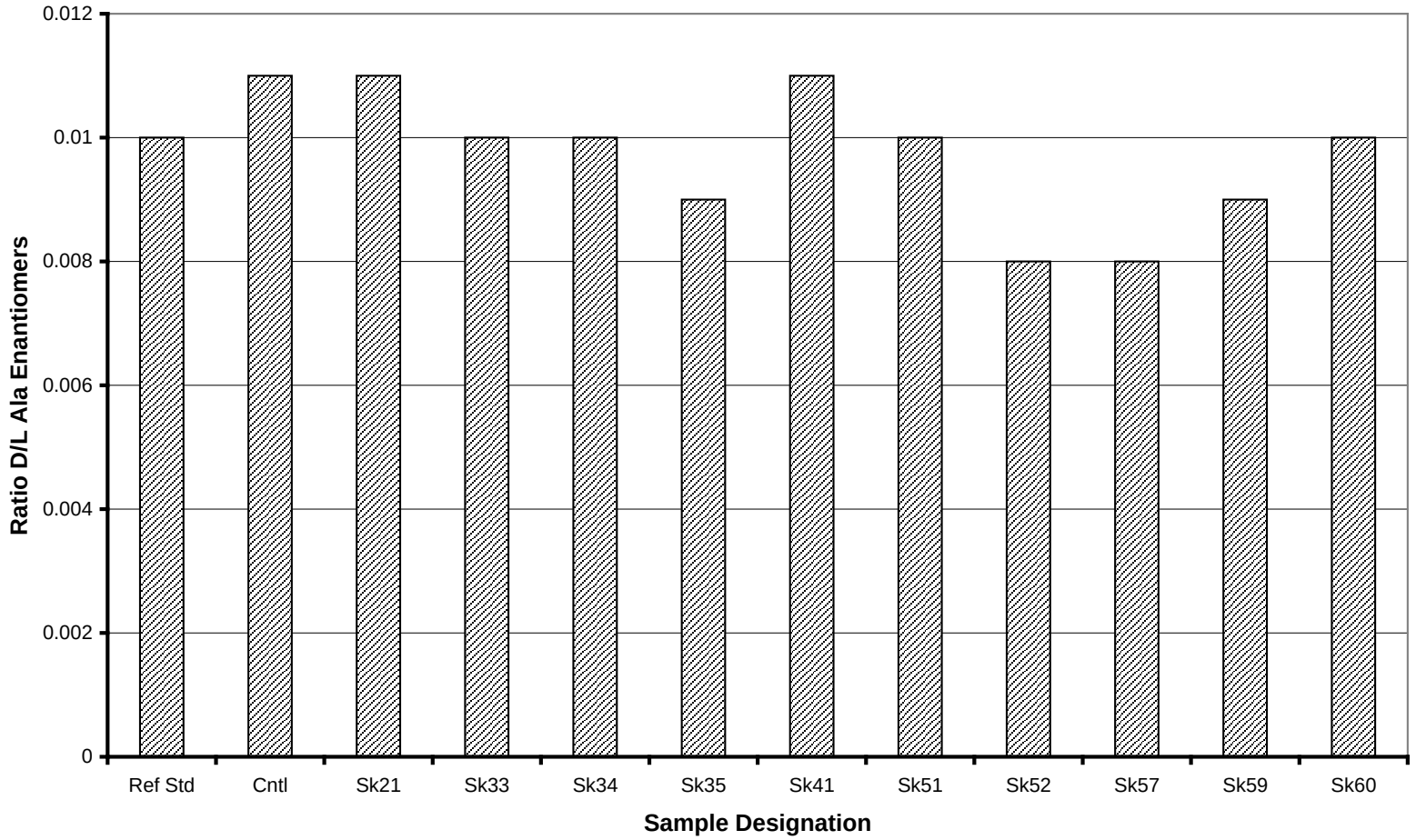


Figure 3.4: Alanine Racemization in Towyn-Y-Capel Population Samples. Barchart compares the D/L ratio of Ala in the sampled skeleton teeth (Table 3.1), bleached tooth control and reference standard. Notice that compared to Asp racemization (Figure 3.3), ratios are very low. Unexpectedly high values would indicate anomally. Sampled teeth from Sk 52 and 57 show low racemization compared to refernce standard.

3.3.2 Determination of Trace Element and Strontium Isotope Concentrations in Towyn-Y-Capel Samples

Table 3.2 provides an overview of the element composition of tooth samples from the Towyn-Y-Capel population, compared to local modern tooth samples and the range of elements usually found in healthy teeth. Elements such as Cd and Pb are present at particularly low levels, with an average that is far below minimal expected values whilst Cu, Sn, Sr and Zn are found at levels that are minimal for a healthy individual (Curazon, 1983). Mn, Mg and Fe are notable in that the values for these elements within the population are significantly inside the expected healthy range. Further analysis of each element and their distribution with samples are provided in figures 3.5-3.11.

Magnesium (^{24}Mg) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Magnesium is one of the most abundant of the trace elements in the human body with particularly high levels found in calcified tissues, due to its involvement in tissue mineralization. Mg levels are normally over 1000 ppm in tooth enamel, and with levels ranging between 2741 and 4412 ppm (Figure 3.5). These levels are within the healthy range and are comparable with both the animal site control (2959 ppm) and the modern control (2821-4826 ppm). According to the literature, the concentrations of Mg and Zn in human teeth show an intimate relationship with their availability in local soil. Levels of Mg are quite low in the surrounding soil at the site at 2276 ppm, which means that either the human tooth actually preferentially accumulates the element, or that Mg concentrations in the surrounding area show large variations. Either case could be true, considering the chemical similarity of Mg to Ca and its natural affinity for substitution along with the high levels of Mg within the inorganic matrix of the enamel and, in addition, the highly variable geology of the site. A recent study on teeth in children from the UK found that Mg levels are considerably higher with an average of 6100 ppm, ranging between 4390-8150 ppm (Brown et al., 2004).

This data clearly show that Mg bioaccumulation levels are lower in the locality of Towyn-Y-Capel, possibly reflecting the differences between pre-industrial levels of heavy metals in the human body as compared to modern levels (§1.3.3).

Manganese (^{55}Mn) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

The levels of Mn in the ancient samples is variable (Figure 3.6), it can be seen from figure 3.6 that for 60% of the samples Mn concentrations are within a narrow range of between 1.498 and 2.177 ppm. Within the next 20%, Sk33 and Sk52 show higher Mn concentrations at 4.471 and 6.372 ppm but these are within the expected range of 1-10 ppm. The remaining 2 samples within this 20% are outliers with Sk21 just outside the range with a Mn concentration of 11.253 ppm and Sk41, which is double the normal Mn concentration at 27.565 ppm. Despite high Mn levels in the soil (137.407 ppm) Sk41 is the only sample with such a elevated level of Mn. Excess Mn in the diet is an unusual occurrence, usually associated with industrial or foundry work, and has been linked to various nervous system disorders ((Normandin & Hazel, 2002).). The results are wel within the modern levels albeit unexpectedly high in a pre-industrial society, perhaps indicating high exposure to natural source.

The Mn concentration found in the tooth enamel from the site samples is over twice that reported in the literature (Kamberi et al., 2009). Although there are no specific studies dedicated to investigating Mn toxicity levels in human dental enamel, the results in this study clerly show elevated levels in a pre-industrial society. It is possible that the high levels of Mn observed in this study could be associated with Mn staining that has been reported in the literature (Stermer et al., 1996), although it seems likely that such discoloration would have been visible in the taphonomic investigation, which was not the case. These high Mn levels may also be an unusual diagenetic modification or crystalline absorption of Mn found in the soil, as levels of the element in the surrounding soil are very high. It has been reported that Mn levels are linked to Fe level, which would be of interest, particularly with reference to Sk41, which may be related to environmental element concentrations, bioaccumulation and preferential affinity.

Iron (^{56}Fe) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Fe levels are highly comparable to the levels of Mn. The Fe levels of Towyn-Y-Capel samples are relatively low, as shown in table 3.2, where Sk34 and Sk35 can be seen to have less than the minimum amount of iron in their diet. Normal Fe levels run from 10-100 ppm, (Curzon,1983) and 40% of the Towyn-Y-Capel population have Fe levels just below that (6.290-9.975 ppm) whilst another 40% have Fe levels just above (10.498-20.858 ppm).

Outliers within the population sample include Sk21 at 32.388 ppm, and Sk41, shows a very high level of Fe at 59.163 ppm (Figure 3.7), which show correspondingly high levels of Mn (Figure 3.6), although well within the healthy range for Fe. However, it is anomalous compared to the population and the controls. Two interesting observations are that there are high levels of Fe in the soil (4517 ppm) although this is not reflected in the population or the proportionally, compared particularly to Mn, to the concentration of Fe present in Sk41, which would lead to some debate regarding whether or not there has been diagenetic change that may explain the high levels due to grave specific diagenetic uptake. Secondly, the level of Fe in the local modern controls reflects the low overall Fe levels in the Towyn-Y-Capel population (5.939-8.125 ppm).

Copper (^{65}Cu) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Cu levels have a similar profile to those of Fe and Mn apart from the high concentration of the metal in the site control (Figure 3.8), which could possibly be a result of the herbivore diet, results show that that levels of Cu in Sk41 are not elevated compared to the remaining Towyn-Y-Capel population. Compared to modern samples, where Cu concentrations vary from 1-10 ppm, concentrations of the metal in the local population appear to be extremely low, ranging from 0.695 -1.517 ppm, although these levels are consistent with those of the modern local samples (0.636-0.703 ppm).

Zinc (^{66}Zn) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Zn levels in the ancient population appear to be relatively consistent At the lower level of Curzon's expected concentration scale for Zn (100-1000 ppm) (Curzon,1983), levels of the metal in 80 % of the Towyn-Y-Capel population lie within 103.19 and 161.04 ppm (Figure 3.9). Sk34 and Sk35 have lower levels of Zn, at 73.76 and 90.14 ppm, respectively, which is consistent with the very low levels of other trace elements found in this sample. Zn levels in the Towyn-Y-Capel population are, however, consistent with reported pre-technological levels of between 60-219 ppm. Zn levels in the animal site control, which was a horse tooth, at 34.60 ppm, and those in the site soil, at 15.07 ppm, appear to be very low compared to the population. The low concentration of Zn in the soil is also interesting, particularly compared with the levels of the metal in the skeletons. This comparison clearly shows a preferential biological accumulation of Zn in these skeletons or an alternative source of Zn found in teeth.

Strontium (^{88}Sr) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Sr levels within the ancient population are reasonably consistent with values between 84.29-178.54 ppm (Figure 3.10). These values are comparable to those of the modern control and are within the lower limits of the expected concentration range for the metal. The animal site control was high with Sr levels at 390.07 ppm, which reflects the high concentration of the metal in the local soil (473.53 ppm).

Cadmium (^{114}Cd) and Tin (^{124}Sn) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Cd and Sn concentrations were generally very low. In particular, Cd levels were less than 0.02 ppm, which is well below concentrations expected for the element. These levels of Cd are also below expected pre-technological levels, which are *circa* 0.1 ppm, and temporally adjacent Romano-British levels, which range from 0.28-0.95 ppm (Whitaker & Stack, 1984; Dobrovalskaya, 2005). Sk41 was the only sample with detectable levels of Cd, at 0.024 ppm, although this is consistent with the anomalously high results obtained for this sample in relation to Mn and Fe levels (Table 3.2).

It may be that the results for levels of Cd in the Towyn-Y-Capel population are influenced by the surrounding environment, as extremely low levels of the element were also present in the animal site control, the modern human control and the soil control. These results would seem to suggest that Cd is rare in the surrounding soil and hence in the food chain, although further research into the bioaccumulation of the element within the area is required to confirm these observations.

Sn concentrations were also extremely low in the Towyn-Y-Capel population with levels generally below 1.0 ppm. Exceptions were the samples, Sk33 and Sk34, with Sn levels of 2.029 ppm and 1.657 ppm respectively. These latter Sn levels are comparable to those of the soil, which were 1.988 ppm. It was also found that the animal site control had Sn levels of 33.95 ppm which was the highest level observed in this study whilst modern control teeth ranged in Sn levels from zero to 15.39 ppm.

Lead (^{208}Pb) Concentrations in Dental Enamel from Towyn-Y-Capel Samples

Pb levels were generally low, well below Curzon's modern scale of expected values (Table 3.2), which is consistent with a pre-industrial environment where levels of the element in skeketons are generally accepted to range between 0.5-1.0 ppm. According to Budd (2006), prehistoric levels of skeletal Pb ranged between 0.04-0.4 ppm and 80% of the Towyn-Y-Capel population samples are within this range. The notable exception to this result is sample, Sk56, which has over six times the Pb concentration of the remainder of the samples at 3.006 ppm (Figure 3.11). High pre-industrial levels of Pb have been reported, particularly in a Romano-British population, where levels of the element up to 40 ppm have been reported although these levels are normally associated with mining (ibid). Pb levels in the modern human controls ranged from 0.34 -21.67 ppm, and although these levels are variable and generally over 20 times higher than those of the Towyn-Y-Capel population, they are representative of the Pb levels that modern humans are exposed to (Curazon, 1983).

^{66}Zn and ^{88}Sr Concentrations in Selected Towyn-Y-Capel Samples

Comparison of ^{66}Zn to ^{88}Sr levels shows an interesting inverse relationship Zn levels in the local soil were 15 ppm, which is very low, compared to those of the all the teeth samples with the closest being the Zn levels in obligate herbivore horse tooth found at the site (Figure 3.12). Modern human control and other teeth samples vary from 73 to 161 ppm of Zn, which may indicate that the accumulation of Zn in animals in relation to surrounding soil types may rise, indicating a preferential biological uptake of Zn or an alternative source of Zn in the diet.

In contrast, Sr levels were generally high with levels of the element in the local soil at 474 ppm, followed closely by those in the obligate herbivore at 390 ppm. However, Sr levels in the medieval population and controls were greatly decreased, ranging between 83 and179 ppm. There appears to be an inverse relationship between the Sr and Zn levels, which could indicate a specific relationship between the two elements or competition in their biological uptake.

Table 3.2: Elemental Concentrations of Towyn-Y-Capel Samples.							
Trace Element	**Modern Tooth[1]**	**Ancient Teeth from Towyn-Y-Capel[1]**					**Healthy Range in Modern Teeth[1,2]**
	Mean	**Mean**	**High**	**Low**	**SD**	**ρ [3]**	
^{24}Mg	3679	3542	4412	2741	638.7	0.848	>1000
^{55}Mn	0.921	6.097	27.57	1.498	8.145	0.076	1 to 10
^{56}Fe	6.793	18.39	59.11	6.290	16.20	0.052	10 to 100
^{65}Cu	0.674	0.970	1.517	0.695	0.280	0.009	1 to 10
^{66}Zn	138.9	119.7	161.5	73.76	27.80		100 to 1000
^{88}Sr	82.48	142.8	178.5	84.29	30.84	0.397	100 to 1000
^{114}Cd	<0.02	ND	0.024	<0.02	N/D		1 to 10
^{124}Sn	<1	ND	2.029	<1	N/D	0.082	1 to 10
^{208}Pb	7.890	0.521	3.006	0.098	0.881	0.265	10 to 100

Table shows mean elemental concentrations of a selection of trace elements in Towyn-Y-Capel samples and control tooth. Analysis of population results including the range and .standard deviation from the mean of the ancient samples and student's *t*-test against modern samples. Right most column shows the expected healthy range of concentration of each element according to the literature.[1]Concentrations in ppm, [2] Reference range from Curzon, 1983.[3]Two tailed ρ value from Two sample T-test assuming unequal variance between ancient population (n=10) and modern (n=3). See relevant figures for degrees of freedom (range between 2 and 10).

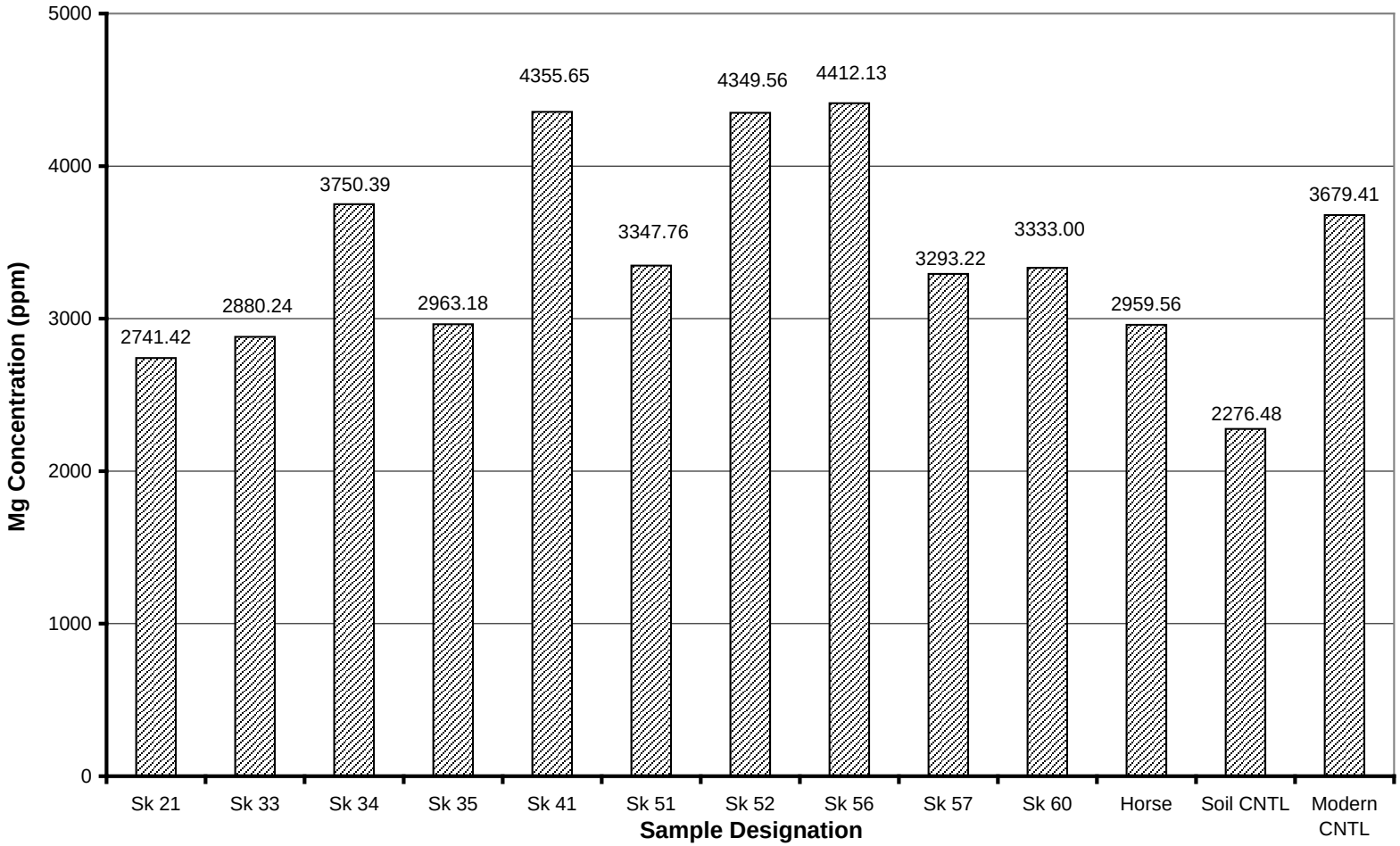


Figure 3.5: Magnesium Concentrations from Selected Towyn-Y-Capel Samples. Two sample *t*-test performed assuming unequal variance between ancient population (n=10) and modern (n=3) with 2 degrees of freedom gives a two tailed ρ of 0.848.

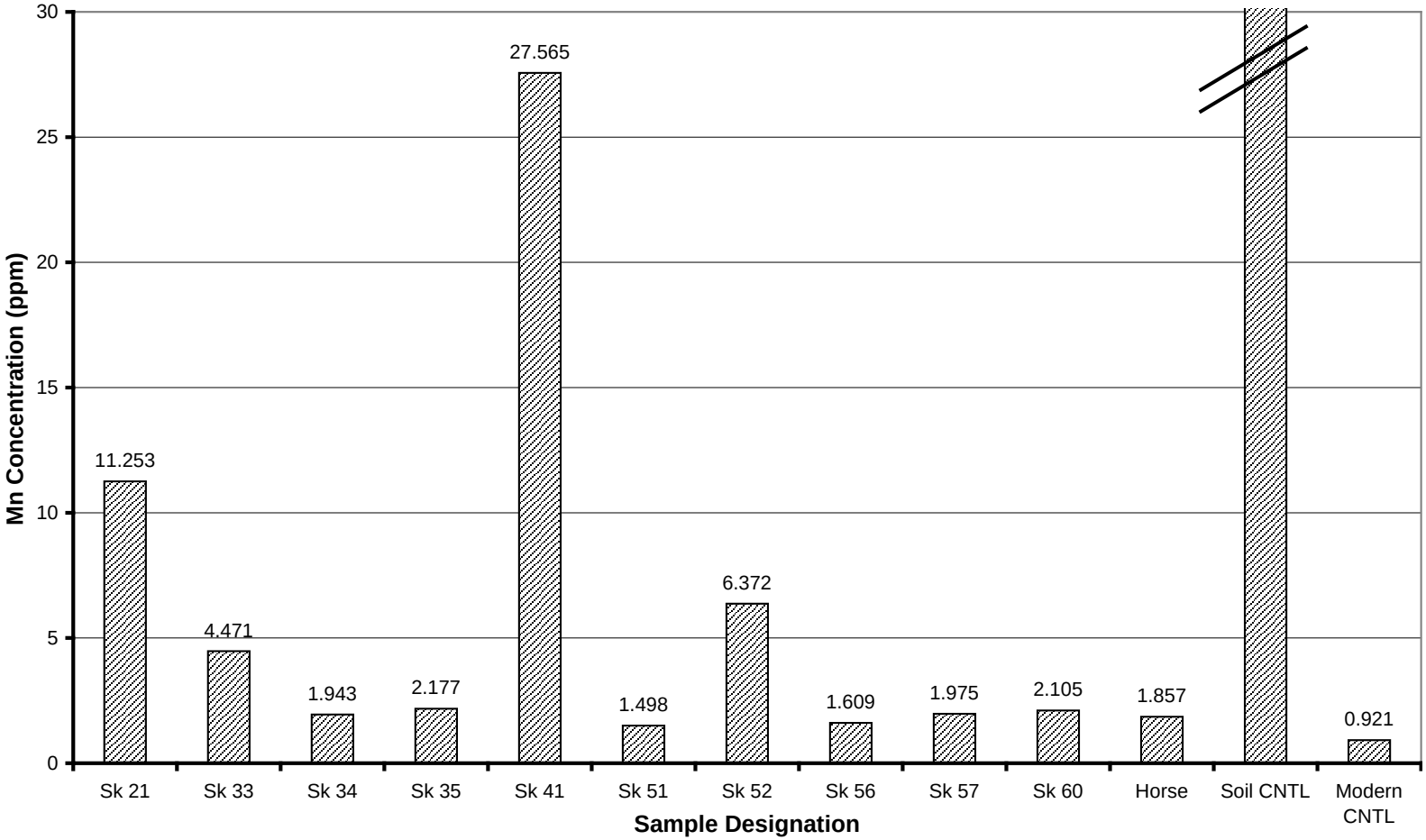


Figure 3.6: Manganese Concentrations from Selected Towyn-Y-Capel Samples. Soil Control Mn level off scale at 137.407 ppm. The anomalous result from the Sk41 tooth sample is of particular interest. Two sample *t*-test assuming unequal variance between ancient population (n=10) and modern (n=3) with 9 degrees of freedom gives a two tailed ρ of 0.076.

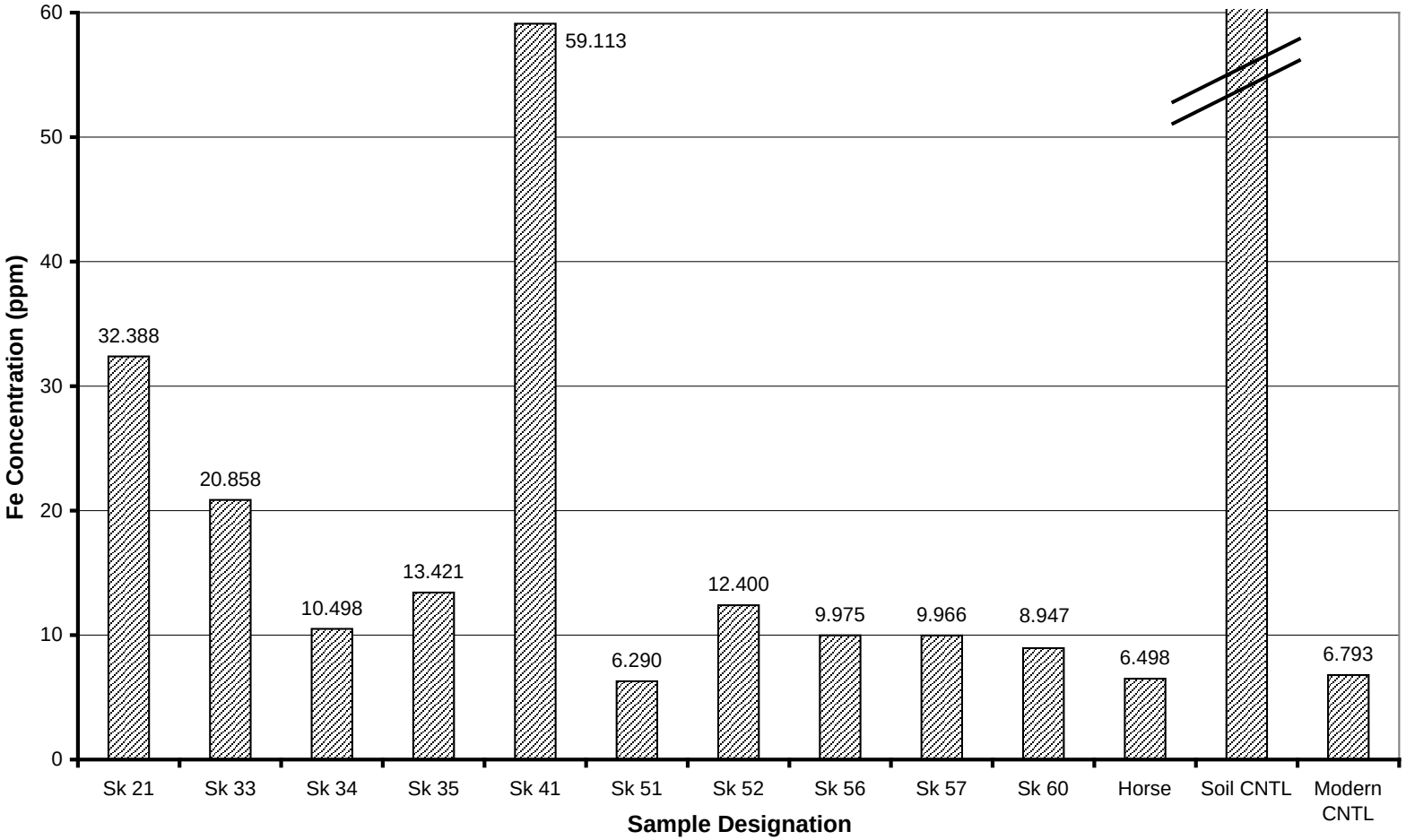


Figure 3.7: Iron Concentrations from Selected Towyn-Y-Capel Samples. Soil control Fe level off scale at 4517.913 ppm. The anomalous result from the Sk41 tooth sample is of particular interest. Two sample *t*-test performed assuming unequal variance between ancient population (n=10) and modern (n=3) with 9 degrees of freedom gives a two tailed ρ of 0.051.

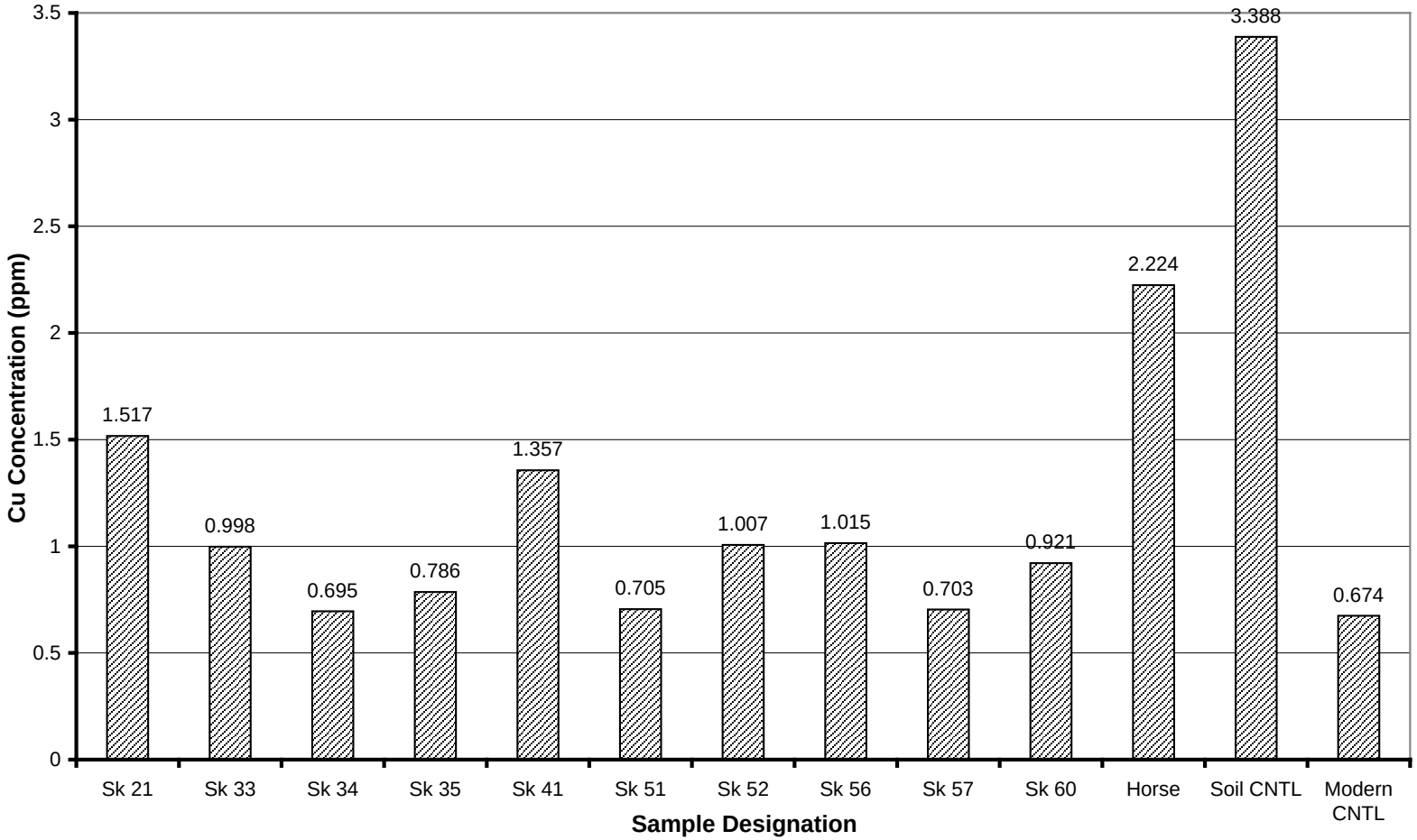


Figure 3.8: Copper Concentrations from Selected Towyn-Y-Capel Samples. Two sample t-test performed assuming unequal variance between ancient population (n=10) and modern (n=3) with 10 degrees of freedom gives a two tailed ρ of 0.009.

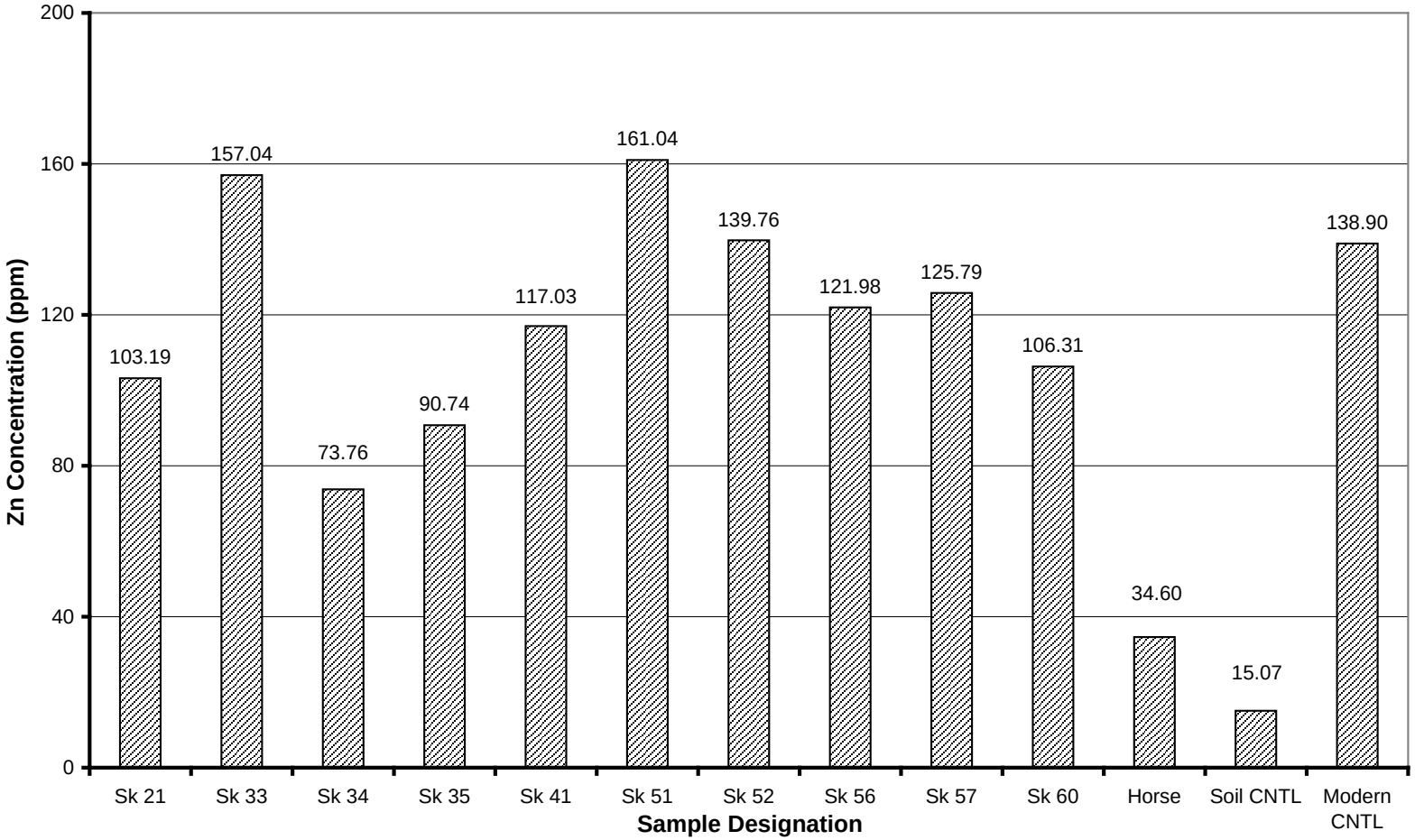


Figure 3.9:_Zinc Concentrations from Selected Towyn-Y-Capel Samples. Two sample t-test performed assuming unequal variance between ancient population (n=10) and modern (n=3) with 4 degrees of freedom gives a two tailed ρ of 0.264.

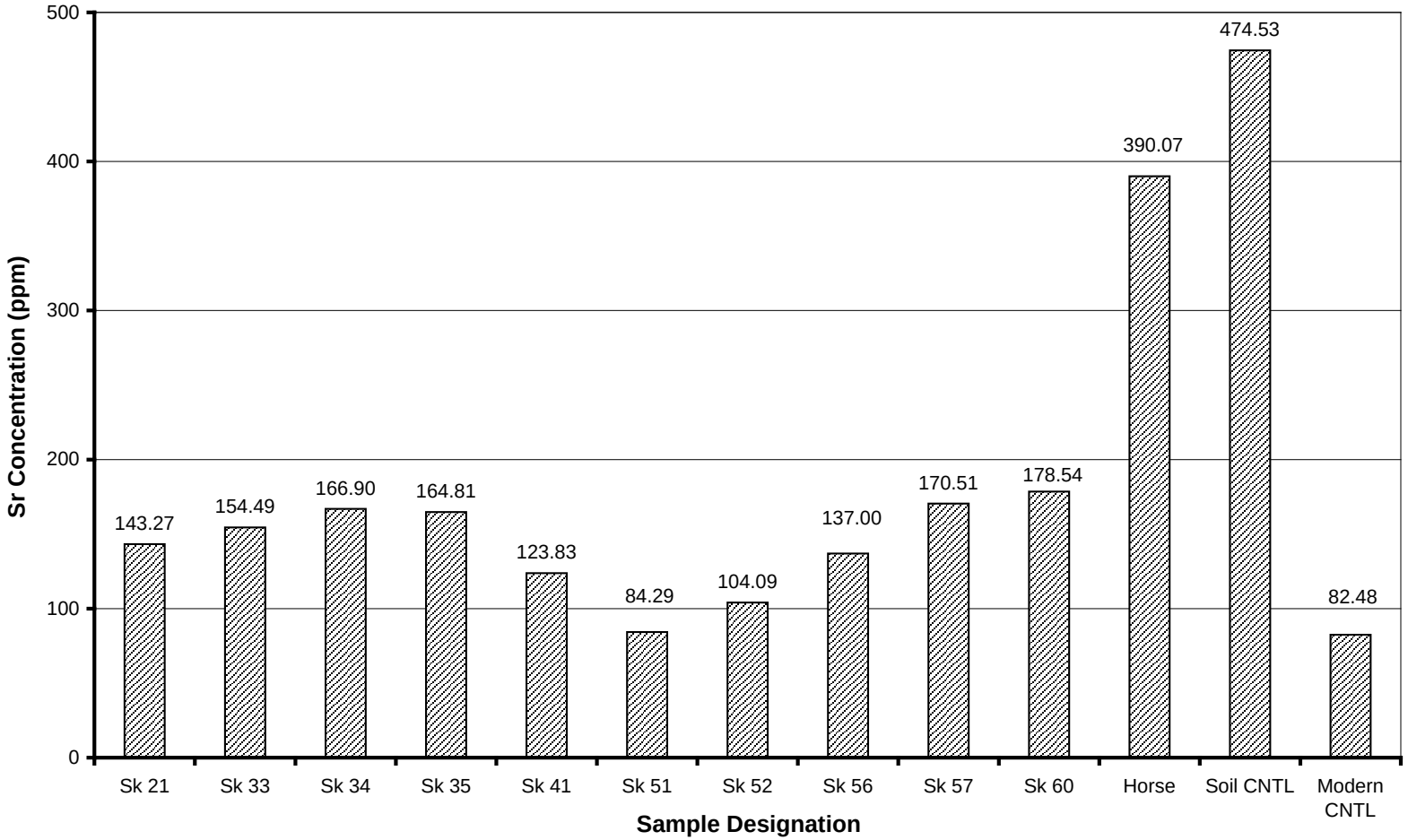


Figure 3.10: Strontium Concentrations from Selected Towyn-Y-Capel Samples. Two sample t-test performed assuming unequal variance between ancient population (n=10) and modern (n=3) with 3 degrees of freedom gives a two tailed ρ of 0.082.

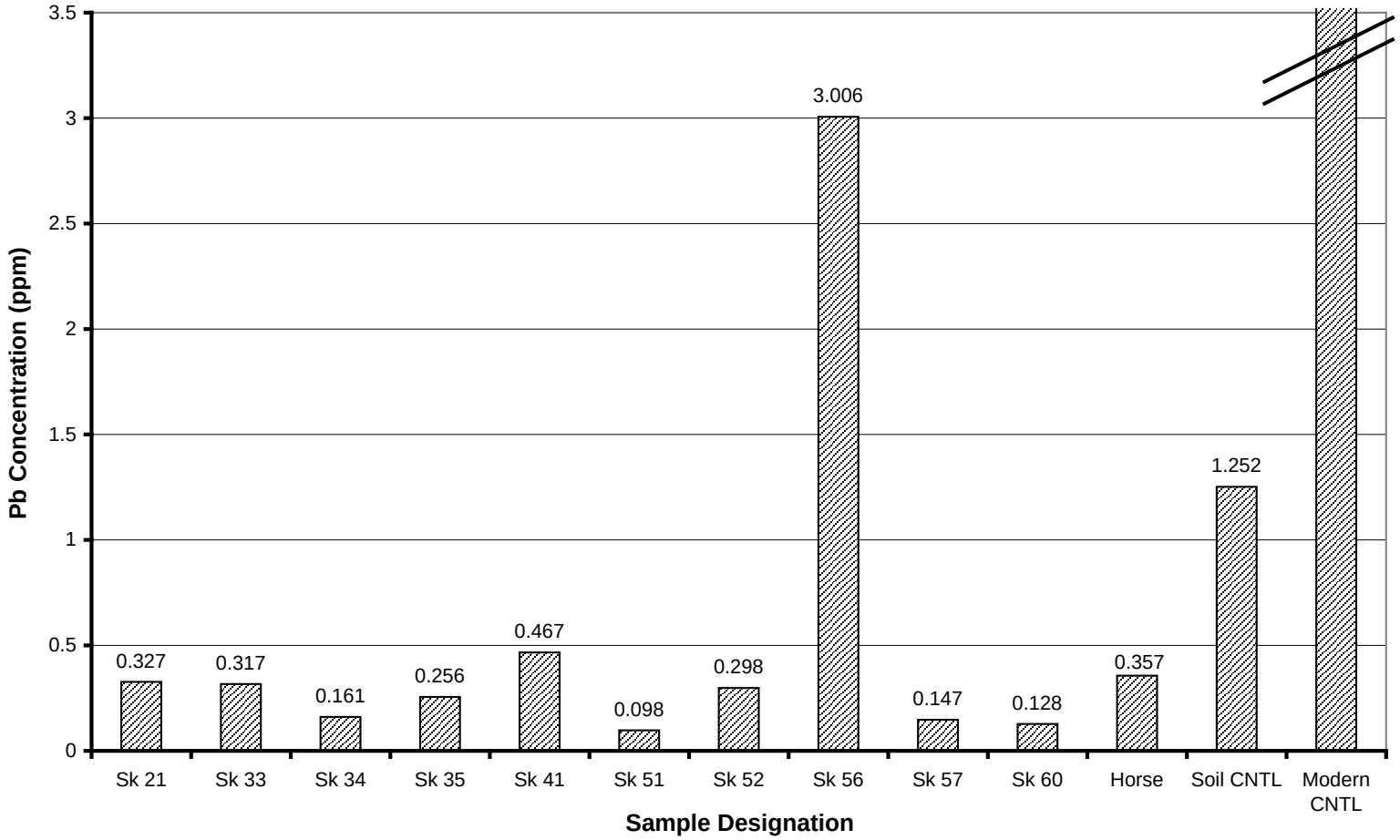


Figure 3.11: Lead Concentrations from Selected Towyn-Y-Capel Samples. Lead levels in Towyn-y-Capel, all low compared to modern (off scale at 7.90 ppm) and far below toxic levels (>100 ppm). Anomlous level (compared to population) in the sample tooth from Sk56 is of interest. Two sample *t*-test performed assuming unequal variance between ancient population (n=10) and modern (n=3) with 2 degrees of freedom gives a two tailed ρ of 0.397.

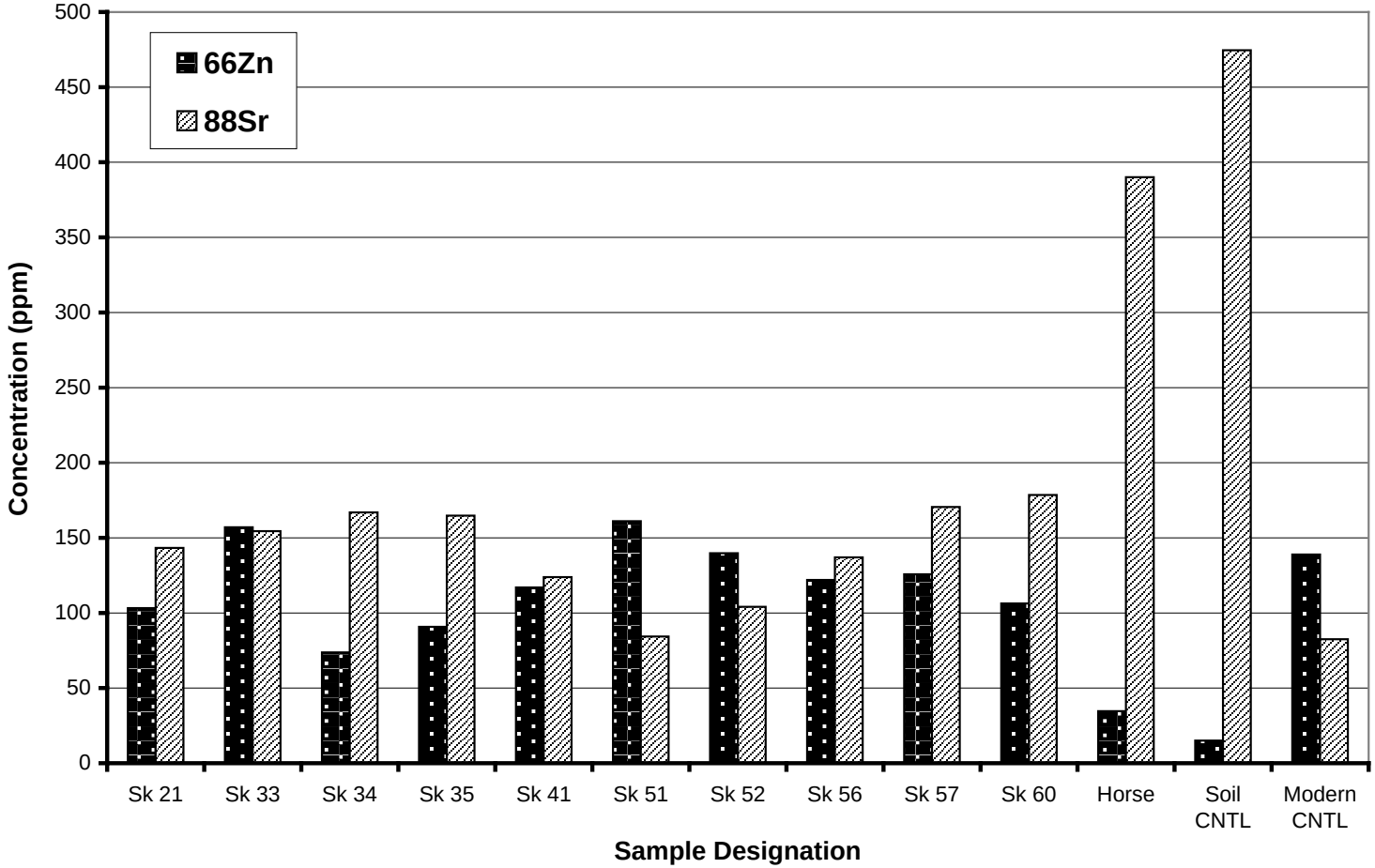


Figure 3.12: Zinc against Strontium Concentrations in Selected Towyn-Y-Capel Samples. Barchart shows the inverse proportional relationship between Zn and Sr in Towyn-Y-Capel human, modern human, herbivore and site soil. Two sample *t*-test performed assuming equal variance between Zinc Concentrations (n=10) and Strontium Concentrations (n=10) of ancient samples with 18 degrees of freedom gives a two tailed ρ of 0.095.

Determination of Isotopic Strontium Ratios of Towyn-Y-Capel Samples

As discussed in Chapter 1 (see §1.3.4; §1.3.5), the highly variable local geology is reflected in the $^{87}Sr/^{88}Sr$ ratio. Values of this ratio, in the area, were expected to be well over 0.710, which is shown in most samples, particularly the human horse and soil controls. The high value of the site soil control is in keeping with the geological age of the local rock strata, which would be expected to yield high strontium ratios. There appears, however, to be several inconsistencies. Firstly, Sk56 has an extremely low Sr ratio, which is below that of water and inconsistent with that of an individual brought up in any part of the British Isles. Secondly, Sr ratios for Sk52 and Sk35 are below those expected for individuals from most of the north west of Wales. Sk52 and Sk35 may therefore may have come from south Wales, or further east, or they may have lived on a predominately fish diet (which is not reflected in the dietary Zn levels).

The individual whose remains compromise Sk35 was most likely not brought up in the Anglesey area, but possibly somewhere else in northern Wales/England, or in an area with similar geology (which varies across the northern Atlantic region). A box has been set around the range of Sr ratios expected from the area of Holy Island-Anglesey. As the area is geographically and geological diverse, a broad range of these ratios is to be expected, although it must be born in mind that biologically available Sr results from mixed rocks pulverized from local geological sources becoming available to the plants and animals from a broad reaching area forming the diet of the local people. In addition, there will be local food imports and exports containing the element, which may serve to normalize the observed strontium ratios in the local population.

The expected Sr range for local samples is between 0.711 – 0.716, which is a wide range and is indicative of relative levels in the soil and the diverse local geology. Sk33 and Sk60 seem to have particularly high Sr ratios at 0.7179 and 0.7181 when the local soil control sample is taken into consideration.

These samples may be either local individuals with a very high strontium ratio, for example from an area made up of predominately pre-Cambrian sediments or they may have come from an area with even older geological formations, such as areas in Southern Norway (Aberg *et al*., 1998) or Greenland (Hoppe *et al*., 2003) which have some of the oldest surface geological formations in the world.

Extremely high or low levels of Sr are known to influence an accurate Sr ratio determination. The grouping of the enamel samples, in terms of Sr level and ratio tends to support the credibility of the results. The two main outliers for Sr ratios, the animal control and the soil control, are expected (Figure 3.14). The soil, as the predominant source of non-biological strontium, will exhibit a high strontium ratio (§1.3.5). The animal control (obligate herbivore) also has a high Sr ratio, which is expected due to its dietary proximity to the source of the biologically available Sr, as the ratios tend to decrease somewhat from the original geological source.

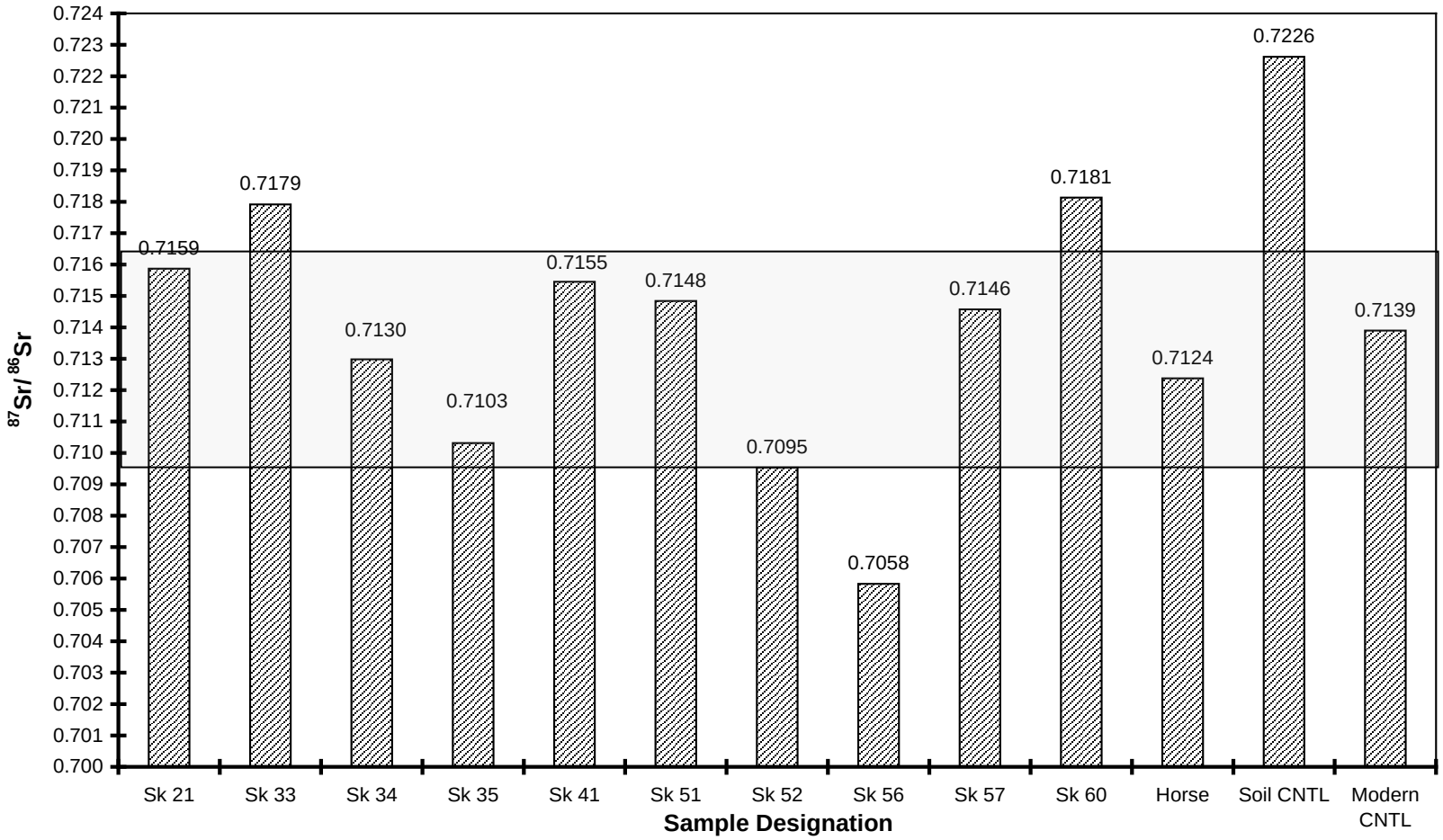


Figure 3.13: $^{87}Sr/^{86}Sr$ Isotopic Ratios of Towyn-Y-Capel Samples. A simplified view of the Strontium ratios of the individuals at the site, with human, site and animal controls. Enclosed area represents expected Sr ratios from controls and local geology (0.710-0.7160). Sampled teeth from Skeletons 56, 33 and 60 stand out as particularly low and high ratios.

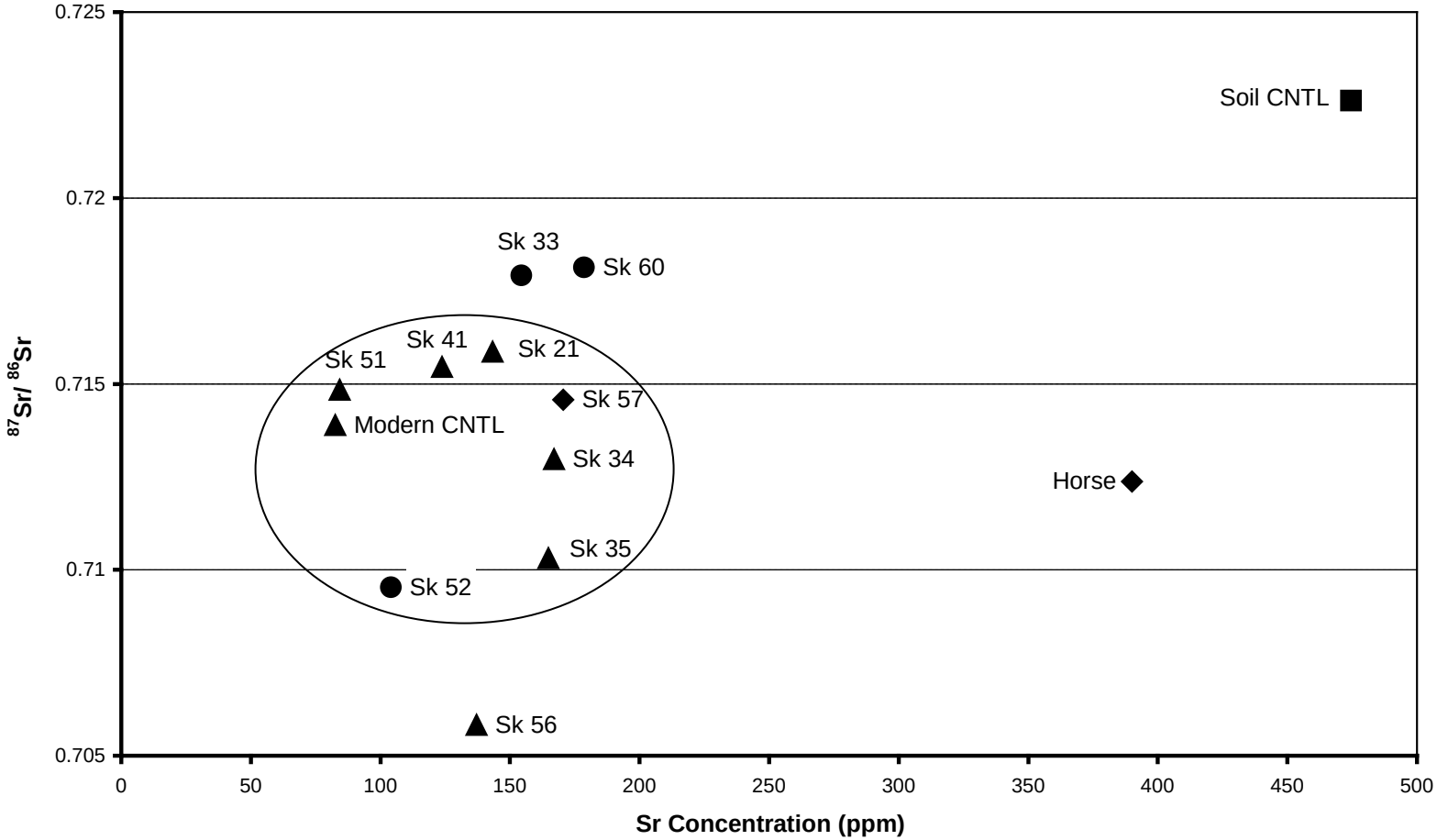


Figure 3.14: Strontium Ratio against Strontium Concentration of Towyn-Y-Capel Samples. Standardised view of $^{87}Sr/^{86}Sr$, comparing Sr ratio and concentrations. Open spherical grouping represents samples within the expected biologically available strontium ratio and concentrations for human Towyn-Y-Capel samples. Apart from the expected oultiers (Horse and soil), sampled teeth from skeletons 56, 33 and 60 lie outside the expected local grouping. Data point shapes represent the sex of the skeleton sampled, Female-circles, Male-triangles, Unknown-diamonds and soil-square.

3.4 Conclusions

3.4.1 The Biological Faction:- Amino Acid Racemization & the Protein Preservation

Indications are moderate for DNA preservation, in that the D/L Asp ratio is below the 0.1 a DNA threshold in all cases, which means there is a potential for aDNA preservation, although most samples are very close to the recommended threshold. This indicates that there is a large degree of degradation of the biological faction. One sample (Sk51) had high levels indicating either exogenous amino acids or relatively greater degradation of the specimen. The absolute levels of amino acids in each individual specimen were also surprisingly high. Furthermore, the concentrations within unknown specimens were higher than in the modern "reference" tooth samples. This leads to some conjecture regarding the validity of the samples although this could occur if the unknowns contained a higher percentage of dentin and the reference contained a higher percentage of enamel.

3.4.2 The Inorganic Faction:- Trace Element Concentrations and Strontium Ratios

Analysis of trace elements is shown in table 3.2. Zn, Cu, Fe are essential elements that are frequently deficient in diets (Brown *et al.,* 2004). Deficiency may cause a wide spectrum of functional consequences. Essentially, the trace element levels in the Towyn-y-Capel site teeth were low and sometimes outside of the detection limits of the ICP-MS. The healthy concentration range of trace elements in adult tooth enamel is shown in table 3.2 as the reference teeth range for each element (Curzon & Cutress, 1983).

From the reference ranges, most of the individuals had trace elements in their teeth corresponding to the lowest elemental concentrations of the modern healthy range. This latter result could indicate a deficiency in the diet or possibly a lack of variation in the diet. It is well established that a lack of fresh fruit and vegetables or low protein foods in an individual can lead to lower levels of Zn in the teeth (Ezzo, 1994). Low Fe levels in teeth are implicated by a lack of red meat or also a diet of high bran (phylates bind and remove Fe), hypoalbuminaemia, phylate and fibre in mainly vegetable diets that can also impair Zn absorption (Brown *et al.,* 2004).

Zn and Cu deficiencies have been reported in humans with protein energy malnutrition (Waterlow *et al.*, 1992), although this cannot be verified by protein content or amino acid concentration without a suitable age and local environment control. As previously discussed, however, the pre-technological level samples tend to have lower overall trace metal levels, although in some cases even compared to these, the trace element levels of the present study are low.

The modern tooth control shown in table 3.2, and figures 3.5-3.11, however, may not be indicative in all facets of the investigation. It been shown that the levels of trace elements in permanent dentition can differ from that of primary dentition, particularly in relation to Sr (Shashikiran et al., 2007). Healthy teeth also have been reported to have higher Sr and Fe content compared to diseased teeth (ibid). It appears that surface adsorption of trace metals in routine daily dental treatments can affect trace metal levels in enamel and in addition, the homogenous nature of the modern diet may affect the local trace element profile and particularly the Sr isotope ratio. The modern tooth control was selected as being more representative local human Sr reference than local fauna, and due to the predominantly non-urban nature of the area, a representative regional control, primary dentition was recommended, due to excellent condition, time span, ethics and ease in sampling.

A number of correlations have been reported between trace element levels in calcified tissues For example: Pb levels, that has a been shown to have a negative influence on Fe, Cu and Zn levels thereby affecting the ratios between Fe/Cu and Cu/Zn (Nowack & Chemternicka, 2000). Relationships have also been shown to occur between Cu andMn (Lapplainen, et al., 1982) and an inverse relationship between Sr-Pb (ibid). Trace element concentrations in the Towyn-Y-Capel population were generally low and coupled with the limited population sampled may affect the detection of putative relationships between the levels of these elements.

Generally, however, there appears to be a loose correlation between the levels of Cu, Mn and Fe, shown principally by the similarity of their profiles across the sampled population (Figures 3.6-3.8). An inverse trend was not observed between Sr and Pb levels, although a previously unreported inverse trend between Zn and Sr levels was detected in most samples, including the controls and appears to have some inverse relationship to the soil concentration (Figure 3.11). This correlation may be related to the adsorption characteristics of the trace metals being assimilated. Zn may compete with Sr in replacing Ca in hydroxyapatite, part of a further

correlation with other trace elements that hasn't been detected or may possibly imply *post-mortem* Sr absorption (Budd et al., 2000)

This investigation into the Towyn-Y-Capel site has unveiled an unprecedented distribution of Sr values for one burial site in Britain (Evans, 2006). The distribution of Sr results showing the putative Holyhead range are compared to Sr levels in figures 3.12 and 3.13. Comparison of these results with table 6.1 gives an estimation of Sr assimilation in the geographical area as indicated by the $^{87}Sr/^{86}Sr$ ratio. Approximately 50% of the individuals analyzed fall between the middle Sr ratio range of 0.712 and 0.714 (highlighted in Figure 3.12), which corresponds to both the controls and to the local geology. Biogenic Sr ratios are usually lower than the surrounding matrix, which corresponds to the local soil Sr ratio of 0.7226 and the gneisses of around 0.7300 previously recorded by Davies (1984). The highest Sr ratios obtained, which were 0.7181 and 0.7179 for samples Sk33 and Sk60 respectively, correspond to values normally found outside of the British Isles. These female individuals may have migrated from an area of dominated by ancient geological formations, such as those found in Norway or Greenland, which are amongst the oldest rock formations on earth (Kawai et al., 2007).

Female migrants from the Nordic countries may have some marital migration implications, as given patriarchal inheritance it has been suggested that female residence patterns in general are more widespread (Gerstenberger et al., 1999). The two samples with Sr ratios close to 0.710 (Sk52 & Sk35 {Figure 3.12 & 3.13}), correspond to the expected values for this ratio in mainland Britain, as can be seen in figure 6.1. The most surprising result from the Sr ratios was that of 0.7058 for sample Sk56, which was the lowest Sr ratio obtained. Such Sr levels are very unusual in the northern Atlantic area, and unique in the area to the geologically young volcanic island of Iceland. The implications of these results is that at least one first generation Nordic individual may have been buried with the local population, and hence possibly lived amongst these people.

In conclusion the results of the study in this chapter show that the although degraded, the organic faction of the teeth, as determined by AAR, is sufficiently preserved, and low in contamination , for the possible biological investigation of both protein and DNA. With regards to the inorganic faction, the trace elements analysis shows no nutrient deficiency or hazardous element exposure. Strontium analysis supports the conclusion that a significant portion of this population was not from the immediate area.

CHAPTER 4

PROSPECTING FOR ANCIENT DNA IN HARD TISSUES FROM TOWYN-Y-CAPEL

4 PROSPECTING FOR ANCIENT DNA IN HARD TISSUES FROM TOWYN-Y-CAPEL

4.1 Introduction

Isolation, amplification and analysis of ancient DNA from calcified tissues is a challenging prospect. Chapter 1 (§1.4-1.5) of this study establishes that when aDNA is present in archaeological material, it is invariably in a poor condition, especially as it is at trace levels compared to modern cells. Apart from the trace quantity it is also associated with a variety of contaminants and inhibitors. To extract this ancient DNA, it is relevant not only to break down the mineralised structure, isolate it from the collagen matrix, as in modern samples, but delicately strike a balance between the purification of the DNA from other organic molecules that may affect latter amplification, but still retain enough of the fragmented DNA for the analysis itself to work. These complex situations are rarely found in normal genomic extraction for modern DNA, except when isolating microbial DNA from soil or faecal material, which involves elaborate purification procedures. However, the target DNA is in a far better condition and quantity that is normally found in archaeological material. It is here that a technical dichotomy occurs in ancient DNA studies. Around the low quantity and quality of the DNA, and the potential for contamination and inhibition, it is clear that one needs to minimise the steps involved in DNA extraction, as each procedure introduces further potential for DNA loss and contamination. However, due to the complex nature of the surrounding biological and mineral matrix, significant purification steps must be taken. It is the careful balance between these two factors that allows successful DNA extraction for molecular amplification. The aim of this chapter of the study is to elucidate the best technique for extraction and amplification of the genetic material from the hard tissues from Towyn-Y-Capel.

4.2 Methods: Laboratory Setup and Contamination Control

From the onset, to minimise human contamination of samples, strict procedures were implemented in the design of this research, from the sampling to laboratory and consumable requirements. In order to accomplish this work, a laboratory was specifically created to avoid possible contamination by amplified DNA or other sources of contamination. Therefore, a pre-PCR laboratory was set up on a separate floor, away from any and all genetic activities and with a double door barrier and restricted access.

This was used for pre-PCR activities which included calcified tissue cleansing, manipulation, sectioning or pulverisation, extraction, purification and PCR setup. In effect, this was a "clean" room, where strict procedures were adhered to in order to minimise human genetic contamination and to keep amplicons from the amplification process and post -PCR manipulation (such as profiling and sequencing of human DNA) separate from the pre-PCR laboratory.

4.2.1 Pre-PCR Laboratory

The physical separation of pre and post-PCR procedures is essential in handling ancient DNA, as contamination from modern DNA or amplicons is a major concern of this research. Modern DNA, is in far better condition than aDNA, and can amplify in preference to the aDNA template. Contamination can occur at any point from sampling to preparation of solution for amplification, and as PCR amplicons can easily be transmitted, even on clothes and gloves, a series of protocols were put into place, including limited human movement. The pre-PCR is a laboratory where samples are taken, stored, cleaned, manipulated, extracted and purified. The pre-PCR setup is also done in the pre-PCR laboratory.

All consumables and solutions were stored, prepared and remained in the laboratory separate to the PCR, post-PCR laboratories. Optimisation of reactions, including controls and other sources of modern and amplified material were kept in PCR laboratories, for use prior to amplification, but physically separated from the pre-PCR laboratories to reduce amplicon contamination. Unfortunately, due to University demand, separate specific aDNA facilities were removed that led to alternative although not ideal laboratory situations, particularly regarding the proximity of separate PCR and pre-PCR facilities, and human movement. The Research was, however, continued, particularly with regard to conformation, at a separate laboratory with physical separation specific for forensic and ancient DNA. The pre-PCR laboratory was kept clean at all times and free from all amplified material. Separate laboratory coats were used for pre and post-PCR areas and gloves were always worn in the pre-PCR laboratory. Within the pre-PCR laboratory separate areas were used dedicated to separate and specific applications for wet laboratory procedures, sample manipulation and photography, sample pulverisation and sample extraction and purification.

4.2.2 PCR Setup

All PCR preparation was undertaken in a dedicated pre-PCR UV cabinet that was cleansed with anti-DNA solution and UV irradiated before, after and occasionally during each PCR protocol. Furthermore to prevent not specific primer binding (primer dimers), all PCR solutions were kept on ice before and during PCR setup. Solutions were pre-aliquoted for each setup where possible to minimize freeze-thawing and contamination. Positive controls were prepared but DNA was not added until the samples are taken to post PCR laboratory.

4.2.3 Post-PCR Setup

Thermocycler use and post-PCR handling was carried out in a separate laboratory physically removed from the pre-PCR laboratory, and dedicated equipment and consumables were allocated for post PCR analysis. The PCR controls were set up (where required) for each reaction, including three negative and one limit of detection positive (referenced standard human DNA, of known concentration and dilution).In addition to the post-PCR solutions and equipment positive controls were also kept in post-PCR laboratory to be added before thermocycling. Thermocycler was preheated to 95^{o}C before receiving reaction tubes, directly from the ice or refrigerator, to minimise the effect of initial temperature ramping.

Protocols to avoid DNA contamination

There were various procedures utilised to minimise DNA contamination. These were instigated into everything from project design, timing of PCR optimisation (or modern aDNA manipulation) compared to aDNA sampling and manipulation, separate storage arrangements for pre and post, genetic and non-genetic material to separate labelling and storage vessels (microfuge tubes) for pre and post reaction material to avoid more than one use and confusion with other material.

Laboratory, material and equipment

All surfaces were cleaned and wiped both with bleach (Kemp & Smith, 2005) and then an anti-DNA solution (halogenated tertiary amine; Microsol, Anachem) before and after use, often with steps involving cleaning, particularly when temporarily leaving the laboratory or in between long incubation steps. During all these cleaning steps, all tubes were closed in order to reduce contamination.

All pieces of equipment employed in this study (centrifuge vortex, incubation chamber, and pipette holders) were cleansed likewise, except for sensitive material affected by bleaching which was cleaned with anti-DNA solution and 70% ethanol solution. In addition to cleansing tube holders, multiple use equipment, including non-UV affected solutions and pipettes, were UV irradiated for 20 min, before and between all pre-PCR manipulations. PCR setup area was cleaned, as mentioned above, and UV irradiated before, after and in between pre-PCR steps as required for at least 20 min.

Where possible, samples were manipulated with pre-sterilised single use or disposable apparatus such as plastic consumables. The entire laboratory was cleaned weekly with 10% bleach, specifically on Friday afternoons, to allow the settling over the weekend of any possible dust or aerosols. Equipment and consumables in the laboratories were only exchanged in a one way system, from pre-PCR to post-PCR laboratories.

Reusable tools were specifically targeted by in a variety of anti-DNA treatments depending on the nature and durability of the material. These included baking at 300^{o}C for over 2 hours (glass and metal ware), sterilisation in a specific “clean only” laboratory sterilisation autoclave, exposure to at least 20 minutes of UV irradiation, soaking or wiping in solution of over 10% bleach, and or cleaning with anti-DNA solution and 70% ethanol. Laboratory movement was restricted and in order to prevent non-determinable contamination, laboratory and material was used exclusively and only by key investigator. All experiments used a limited number of samples, in material manipulation, extraction, and pre-PCR setup, in order to reduce errors and cross contamination between the samples themselves.

Preparation of solution and materials

All pre-PCR liquid handling was done using specific pipettors for each particular use. Separate pipettes were used for extraction, pre-PCR setup, and post-PCR manipulation with single use γ-irradiated filter tips (aerosol barrier tips). Pipettors were subject to regular decontamination in anti-DNA solution, at least once a month unless contamination was suspected, and during use were regularly UV-irradiated as part of the preparation procedures before laboratory use. Reagents stocks and disposable supplies used for ancient specimens were bought in specifically for the use in the pre-PCR laboratories, pre-sterilised where possible, recorded (batch control) and further UV-irradiated on arrival in laboratory.

All consumables were autoclaved (where appropriate), UV or γ-irradiated and/or certified DNA free by Manufacturer, and for each procedure fresh consumable stocks were utilised. Where appropriate, consumables were pre-aliquoted for single use to avoid back contamination.

Use of controls and limited samples

All genetic experiments, from extraction to PCR setup included the use of at least one positive control, a modern DNA, bone or tooth sample, prepared outside of the pre-PCR laboratory, in order to determine whether or not a reaction and an extraction and amplification were successful. Negative controls were also run on all genetic experiments involving aDNA in order to detect contamination. Negative controls were run in triplicate for both extractions and amplifications. In the case of extraction this was either with sterile water when assaying animal bone or tooth, or animal bone and tooth when extracting from human bone or tooth. PCR negatives consisted of normal reaction contents with the template substituted by an equal volume of sterile water). In total each extraction-amplification set consisted of 6 negative controls. For each extraction a maximum of 10 samples were used and each PCR setup (or batch) was limited to 20 or less reactions.

4.3 Source Material for Genetic Investigation

The material used for this study was the sampled calcified tissue remnants from the Towyn-Y-Capel site previously detailed in Chapter 2. For preliminary work, loose and random finds of calcified tissues found at the site were used. These were mostly common faunal remains (Table 2.1) and the occasional non burial associated human material. A list of the samples is given in table 4.1 for loose faunal samples and Table 4.2 for loose human samples. These samples were characterised by the small size of no more than 2-4 cm in diameter, mostly indeterminate origin and variable morphological condition. Samples of patella and teeth used for this research are given in Tables 4.5 (Patella) and 4.6 (Teeth). Selection and analysis were as described in Chapter 2 (§2.2.1). 2nd and 3rd molars were normally selected due to gross condition, size and lack of wear, large protective enamel crown and large potential pulp chamber and therefore theoretically larger proportional number of odontoblast processes.

Table 4.1: Non-burial Associated Faunal Bone Collection used for aDNA Research.

Sample	Find	Context	Sample Description	Putative Species Identification
A0	?	?	Random bone	Unknown
A1	?	?	1 bone fragment	Unknown
A2	74	115	2 vertebra (3 pieces)	Unknown
A3	148	126	Tooth	Horse/Cow
A4	65	115	Talus	Sheep/Pig
A5	99	101	Metatarsal	Sheep/Pig
A6	?	?	Metatarsal	Sheep/Pig
A7	65	115	Random bone	Unknown
A8	?	?	Inominate pneumatic bone	Bird
			Ulna, 2 vertebra	Bird
			Long bone	Bird
A9	214	?	Long bone	Bird
B0	2	101	Metatarsal	Sheep/Pig
B1	68	115	Metatarsal	Human
B2	?	?	Long Bone	Horse/Cow
B3	?	?	Incisor Tooth	Unknown
B4	117	134	Teeth & mandible	Rodent/ Rabbit
			Shoulder blade	
			Pneumatic bones	
			2 scapula	
			2 vertebra	
			5 metatarsals	
			2 phalanges	
			Long bone	
B5	134	151	2 large teeth	Unknown
			Part of skull	Rabbit?
			(S1) Metatarsal	Human
			(S2) Metatarsal	Unknown
			(S3) Phalange	Human
			Fragments	Unknown
B6	99	127	Vertebra	Rodent
			Long Bone	Rodent
B7	99	127	Pelvis	Human?
			Skull fetal?	Human?
B8	519	528	Large Tooth	Horse/Cow
			(S1)2 unidentified bones Possible rib/long bone	Unknown
			(S2) 2 small bones	Unknown
B9	202	101	Vertebra	Horse/Cow/Deer
C0	?	?	Long bone	Bird
C1	?	?	Long bone	Bird
C2	?	?	Shoulder/pelvis	Bird
C3	?	101	Long bone	Bird
C4	?	101	Immature long bone	Chicken?
C5	?	147	Sacrum	Rodent/Bird?
C6	?	147	Unidentified bone	Bird
			(S1) Large bone	Unknown
			(S2) Medium white bone	Unknown
			(S3) Small crescent shaped bone	Unknown

List of the unknown and non-burial associated finds recovered from the archaeological dig at Towyn-Y-Capel site (GAT 1746) and used for aDNA research. Sample ID related to PCR extraction, Find number related to archaeological location at site and context number designates the archaeological stratigraphy of the Site (Soil layer) within which the sample was found.

Table 4.2: Non-burial Associated Human Bone Collection used for aDNA Research	
Sample Designation	**Sample Description**
HA1	Upper molar adult (RM^2)
HA2	Pre molar right ($RPM_{2)}$
HA3	Canine
HA4	Lower (M_1) immature <6
HA5	Deciduous lower molar (roots not completed <2)
HA6	Deciduous child canine (<2)
HA7	Human damaged tooth
HA8	Right ($M^{2 \text{ or } 3}$) very worn, mature >40
HB1	Fragment of Fibula shaft
HB2	Medial phalange
HB3	Immature (young child) phalange
HB4	Fragment of fibula shaft (same as above)
HB5	Scaffold wrist bone
HB6	Proximal fragment of phalange
HB7	Fragment of fibula shaft (adult)
HB8	Fragment of fibula shaft
HC1	Proximal phalange (adult)
HC2	Glenoid cavity of scapula (human?)
HC3	Distimal humorous fragment (adult)
HC4	Cranial vault fragment
HC5	Cranial vault fragment (adult)
HC6	Articulating process of vertebra fragment

Random finds from Towyn-Y-Capel archaeological site. Anglesey, North Wales Context (508) Find (152) Area (20)

Table 4.3: Some Faunal Samples Extracted for DNA analysis			
Sample/PCR No	**Sample Designation**	**Hard Tissue Type**	**Weight (grams)**
0	Control	N/A	N/A
1	A0	Bone Fragment	1.1
2	A2	Vertebra	0.7
3	A7	Patella	1.0
4	B3	Incisor Tooth	1.0
5	B4	Bone fragment	0.8
6	B5 S1	Phalange	1.0
7	B5 S2	Metatarsal	1.1
8	B5 S3	Metatarsal	0.8
9	B7	Disk like bone	1.2
10	C0	Long Bone	1.0
11	C1	Long Bone	1.5
12	C2	Pelvis/Shoulder	0.9
13	C3	Long Bone	1.1
14	C6 S1	Bone Fragment	1.0
15	C6 S2	Crescent Shaped	0.8

An example of the aDNA extract samples and volumes used for the analysis correspond to samples used for RAPD analysis (Figures 4.17-4.25).

Table 4.4: Human Samples Extracted for DNA Analysis			
PCR No	**Sample Designation**	**Hard Tissue Type**	**Weight (grams)**
1	Ex	Patella	0.13
2	HA4	Tooth	0.08
3	Sk17	Patella	0.05
4	HA8	Tooth	0.12
5	HA7	Tooth	0.12
6	HA6	Tooth	0.12
7	HA5	Tooth	0.11
8	SK 1	Patella	0.1
9	Sk511a	Patella	0.1
10	Sk511b	Patella	0.1
11	Sk514	Patella	0.11
12	Sk516	Patella	0.12
13	Sk508b	Patella	0.11
14	Sk508a	Patella	0.1
15	Sk510	Patella	0.16
16	Unknown A	Patella	0.11
17	Unknown B	Tibia	0.12
18	HA1	Tooth	0.14
19	HA3	Tooth	0.11
20	Control	No Sample	N/A
21	Positive	N/A	N/A

Table 4.5: Human Skeletal Patella Samples Extracted for DNA Analysis							
No[1]	**Sk[2]**	**Type[3]**	**Weight[4]**	**No[1]**	**Sk[2]**	**Type[3]**	**Weight[4]**
1	Sk1	Patella	N/D	26	Sk68	Patella	0.23
2	Sk17	Patella	N/D	27	Sk69	Patella	0.24
3	Sk20	Patella	N/D	28	Sk81	Patella	0.34
4	Sk22	Patella	N/D	29	Sk88	Patella	0.17
5	Sk25	Patella	0.11	30	Sk93	Patella	0.33
6	Sk33	Patella	0.14	31	Sk101	Patella	0.13
7	Sk34	Patella	N/D	32	Sk102	Patella	0.32
8	Sk35	Patella	N/D	33	Sk103	Patella	0.37
9	Sk41	Patella	N/D	34	Sk104	Patella	0.35
10	Sk43	Patella	0.17	35	Sk105	Patella	0.29
11	Sk47	Patella	0.23	36	Sk108	Patella	0.18
12	Sk48	Patella	0.25	37	Sk184	Patella	0.5
13	Sk49	Patella	0.31	38	Sk508	Patella	0.15
14	Sk50	Patella	0.36	39	Sk510	Patella	0.21
15	Sk51a	Patella	0.22	40	Sk511	Patella	0.13
16	Sk51b	Patella	0.14	41	Sk514	Patella	0.19
17	Sk53	Patella	0.2	42	Sk516	Patella	0.29
18	Sk54	Patella	0.18	43	SkUK A[5]	Patella	0.18/0.25
18	Sk55	Patella	0.26	44	SkUK B1[5]	Patella	0.28
19	Sk56	Patella	0.19	45	SkUK B2[5]	Patella	0.15
20	Sk59	Patella	0.44	46	SkUK C1[5]	Patella	0.24
21	Sk60a	Patella	0.36	47	SkUK C2[5]	Patella	0.14
22	Sk60b	Patella	0.15	48	HVT[6]	Tooth	0.2
23	Sk63	Patella	0.34	49	HVR[7]	Rib	0.12
24	Sk66	Patella	0.21	50	THG[8]	Tooth	0.11
25	Sk67	Patella	0.18	51	PHG[9]	Patella	0.06

[1] PCR Number, [2] Human Skeleton Number, [3]Bone Type used to sample bone powder, [4] Powder weight of bone in grams used for extraction, [5]Unknown Skeleton, [6] Human Victorian Tooth, [7] Human Victorian Rib, [8] Tooth Human GAT 1746, [9] Patella Human GAT 1746.

Table 4.6 Skeletal Teeth Samples Extracted for DNA Analysis					
No[1]	**Sk[2]**	**Type[3]**	**No[1]**	**Sk[2]**	**Type[3]**
1	Sk1	$RM_{2/3}$	33	Sk60	LM_1
2	Sk4	RM_2	34	Sk63	LM_3
3	Sk7	L/RM[1]	35	Sk64	LM_2
4	Sk11	RM_1	36	Sk67	LM_3
5	Sk13	LC_1	37	Sk68	RM_2
6	Sk16	LM_2	38	Sk69	LM_3
7	Sk17	LM[1]	39	Sk70	LM_3
8	Sk18	RM_1	40	Sk71	LM_1
9	Sk20	RM_3	41	Sk72	RM_3
10	Sk21	LM[3]	42	Sk79	M?
11	Sk21	RM[3]	43	Sk80	LM[3]
12	Sk22	RM_3	44	Sk81	LM[3]
13	Sk24	RM_2	45	Sk82	M[?]
14	Sk25	LM_3	46	Sk85	LM[3]
15	Sk26	RM_3	47	Sk87	I?
16	Sk30	RM_2	48	Sk88	I?
17	Sk33	RM_3	49	Sk89	M?
18	Sk34	LM_3	50	Sk99	M?
19	Sk35	LM_2	51	Sk103	LM_3
20	Sk39	$RM_{1/2}$	52	Sk106	LPM_3
21	Sk41	RM_2	53	Sk107	M?
22	Sk43	$RM_{2/3}$	54	Sk108	RM_3
23	Sk43	RM_1	55	Sk139	RI[1]
24	Sk48	RM_2	56	Sk502	LM[2]
25	Sk50	LM_3	57	Sk508	LM_3
26	Sk1	$RM_{2/3}$	58	Sk511	RM_1
27	Sk50	RM_3	59	Sk512	LM[1]
28	Sk51	LM_3	60	Sk513	RM[1]
29	Sk52	RM_2	61	Sk516	RM_3
30	Sk53	LM_2	62	Sk517	LM[2]
31	Sk56	LM_3	63	Sk517	LM_3
32	Sk57	RM_2			

[1] PCR Number, [2] Human Skeleton Number, [3]Tooth designation used to sample tooth powder.

4.4 Methods: Ancient DNA Extraction, Amplification and Analysis.

Whole Sample Cleansing and Decontamination

As the material for DNA analysis was sampled directly from the burial site, it was first cleansed of site material. This was undertaken in the wet laboratory section of the pre-PCR laboratory as described previously (§2.2.1 &§4.1) where procedures were implemented to minimise modern human DNA contamination. Samples were removed aseptically from sample bags and rinsed in deionised water and cleansed by surface abrasion using a soft brush to remove surface contaminants and then subjected to a repeat wash and rinse cycle of 10% bleach, anti-DNA solution (Microsol, Anachem) and 70% ethanol. For teeth, sterile pumice was also used to assist in surface abrasion. Samples were then catalogued and photographed (§2.2.1) before UV irradiation at 254 nm for 1 hour (30 min on each side). Samples were taken directly from the UV cabinet for surface sanding and bone milling. Limited samples of ten or less were handled on any one day. All cleansed samples were processed on the same day, as they were removed from storage.

Pulverisation

Large bone samples (in particular patella) were placed in multiple sterile freezer bags, snap frozen in liquid nitrogen and crushed into smaller fragments using a sterile pestle and mortar. All samples were transferred (aseptically) to Spex cryovials and thereafter pulverised in a nitrogen mill (Spex Certiprep), using a full impact program of 1.5 min with two 10 second interval. The powdered contents were carefully aliquoted into 3-4 sterile screw tope tubes, the remainder (depending on the size of the sample) were placed in sterile single use falcon tubes. Tooth samples fitted directly into the Spex cryovials and pulverised under the same settings, although in some cases this was repeated more than once due to the hardness of the material (in particular with modern material). Tooth powder was thereafter directly aliquoted into pre-sterilised 1.5 ml screw top cryo microtubes in aliquots of approximately 200 mg. Samples of bone and tooth were stored independently at –15 ^{o} C to –25^{o} C for further use. In the case of all samples, ancient samples were prepared first and independent of modern samples. Modern samples were prepared in a separate laboratory on different days.

4.4.1 The Determination of the Optimal EDTA Demineralization Conditions for Calcified Tissues

Optimal DNA recovery from ancient material required complete demineralization of the sample. Following experiments performed on whole section decalcification performed earlier (§2.2.3), the prevailing methodologies regarding demineralization and the rate of reaction, was placed into question. In order to clarify the situation, a number of assays were conducted in determining the ideal demineralization conditions in order to optimise genetic recovery, particularly at the microfuge tube scale.

Determination of the Rate of Decalcification with EDTA

The rate of demineralization was calculated by performing 3 different assays using EDTA. Demineralization was performed on fresh bone and teeth from *Bos taurus* in order to avoid contamination with modern DNA. Sets of 50 mg of bone and tooth material in 2 ml tube were incubated with a 0.5 M EDTA solution for different time periods, ranging from days to minutes. Each bone and tooth sample was incubated at 37^{o}C over the specified time period, and after incubation the reaction is stopped by a 1 minute centrifugation at 13400 rcf (MSE Microcentaur), in order to condense all the remaining mineralised material and pipette off the supernatant for analysis. In addition to determining the rate of demineralization a subsequent assay looked into the use of short term replacement in order to increase net demineralization after and if the end point is reached in modern tissue samples.

Colourimetric Determination of Calcium Concentrations in Solution

For determining the rate of reaction a suitable end point determination is required, ammonium oxalate in particular is used for this purpose as it precipitates in the presence of calcium ions. A quick assay was developed using this phenomena, and measuring the rate using a 1 ml cuvette in a UV/Vis spectrophotometer to determine the accumulation of calcium into solution, which in turn was ascertained over a broad range of wavelengths from 400-600 nm. A value of 500 µl of the ammonium oxalate solution was added to 500 µl of the demineralization solution in a 1 ml cuvette, inverted and the adsorption measured using a Jenway Genova UV/vis spectrophotometer.

Spectroscopic Determination of Calcium Concentrations in Solution

The direct concentration of calcium in solution was determined by AAS (Atomic Absorption Spectroscopy) through dilution of the heavily calcified solutions by factors of up to 2000. This sensitive technique was undertaken to provide a real approximation as to calcium ions concentration in solution, and thereby a closer estimate to total volume of calcium in solution and in the modern calcified tissues, in addition to providing a basis for the calibration curve required for the colorimetric assay. A series of dilutions were made up for each solution, between 1 in 10 to a 1 in 2000 dilutions. An aliquot of 100 µl of the diluted sample was aspirated into the flame apparatus of the AAS in order to calculate the concentration of calcium in solution.

4.4.2 Procedure for Demineralization for DNA Extraction

Using previously ascertained knowledge regarding EDTA reaction kinetics and content calculations, complimented by personal X-Ray radiography observations, along with best technique the following decalcification protocol was used on all samples.

An amount of 100 mg of bone or tooth powder were weighed into two - 2 ml microfuge tubes (Microfuge tubes and rack were all weighed pre-sterilised and UV irradiated before use {average weight 1.5 ml tubes 0.88-0.94 g, rack 16.61 g} and measurements accurate to 3 decimal places i.e. ±0.001 g). 1000 -1800 µl of soaking solution was added to each tube (0.5 M EDTA, 0.5% SDS and 1 mg/ml proteinase K) and vortexed thoroughly. The tubes were subsequently sealed with parafilm to avoid concretion forming at lid. Samples were incubated, rotating on a low setting at 37 ^{o}C for 2-3 days, in a Micro-4 hybridization oven, and checked periodically to avoid sedimentation. Samples were subsequently used for extraction of DNA. In teeth demineralization and later repeat of bone demineralization 0.1M N-Phenacylthiazlium bromide (PTB) was added soaking solution. For modern samples larger volumes of EDTA were required for the precise extraction of DNA, or more than two substitutions.

4.4.3 Methods for DNA Extraction & Purification

Initial investigations into the fossilised material from Towyn-Y-Capel were performed using Chelex and QIAmp DNA mini-kit on faunal samples in order to use non specific polymorphic nuclear DNA profiling. Following this work, a more specific work was done into a wider range of extraction techniques, in particular commercial kits in order to optimise both DNA quantity and quality. In this assay, a trace quantity of modern human control DNA was added to similar

volumes of animal bones and extracted according to each of the techniques outlined below in order to determine which is the most appropriate for the research.

DNA Extraction using the Chelex Method

Based on the procedure of Walsh et al., (1991), the Chelex method did not involve decalcification. An amount of 200-300 mg of bone powder was added to sterile microfuge tubes, followed by the addition of 200 µl of 5% Chelex® 100 and incubation at 56 ^{o}C for 2-4 hours in a waterbath. Samples were then removed, vortexed for 10 sec and placed in a boiling water bath for 8 min. Samples were removed, vortexed again and centrifuged for 2 min at 13 400 rcf (MSE Microcentaur). Supernatant was carefully removed without disturbing the pellet, placed in a fresh microfuge tube and stored at –15 to –25^{o} C for further use.

DNA Extraction from Calcified Remains using the Geneclean™ ancient DNA kit

The samples were processed using a Geneclean kit for ancient DNA (BIO 101) according to the Manufacturers' instructions with some modifications1 ml of dehybernation solution was added to the decalcified material which was vortexed briefly before rotating for 2 (to 4) hours at 60^{o}C in a Micro-4 hybridisation oven. Samples were removed, centrifuged at high speed (16 100 rcf) for 5 min and the supernatant transferred to a fresh 2 ml tube. A volume of 300 µl of aDNA glassmilk was added and briefly vortexed before rotating at 37^{o}C for 30 min. The suspension was transferred to a spin filter and catch tube and centrifuged at 16 100 rcf for 1 min (repeated as required). Each catch tube was emptied and 0.5 ml of Salton wash solution 1 was added. These were centrifuged 16 100 rcf and each catch tube emptied of supernatant. This procedure was repeated with 0.5 ml Salton wash solution 2 and twice more with 0.5 ml of aDNA alcohol wash. Catch tubes and spin filters were centrifuged for 2 min "dry" and each catch tube was emptied. Filters were placed into a DNA free elution catch tube (fresh tube) and 50-100 µl DNA free elution solution was added to the re-suspended pellet before centrifuging for 1 min to transfer the elution to a catch tube and stored at –15 to –25^{o} C for further use.

DNA Extraction from Calcified Remains using the QIAmp mini blood DNA kit

The QIAmp DNA Mini Kit (QIAGEN) was used according to the Manufacturers' instructions with some modifications. A volume of 200 µl of buffer AL was added to the decalcified sample, vortexed and incubated at 70°C for 10 min. The sample was briefly centrifuged (Microcentifuge,

Eppendorf 5415R) before adding 200 µl ethanol (96–100%) which was mixed by vortexing. The sample was again briefly centrifuged before adding the mixture to the QIAamp spin column, and subsequent centrifugation at 5900 rcf for 1 min.

The excess was discarded and 500 µl of buffer AW1 was then added before further centrifugation again 5900 rcf for 1 min. The excess was discarded before the addition of 500 µl of Buffer AW2. Spin columns were centrifuged at full speed (16,100 rcf) for a further 3 min. The excess was discarded before centrifuging the spin column "dry" at full speed for 1 min. Finally, after discarding the 2 ml tube and excess, the QIAamp spin column was placed into a clean 1.5 ml microcentrifuge tube, 200 µl buffer AE was added and, after incubation at room temperature for 1 min, the sample was centrifuged at 5900 rcf for another 1 min. The spin column was then discarded and the elution stored at –15 to –25^{o}C for further use.

DNA Extraction from Calcified Remains using the Commercial Forensic & Soil kits

Selected samples were processed using the MoBio Forensic DNA extraction kit (MoBio laboratories Inc), MoBio Ultraclean soil DNA extraction kit (MoBio laboratories Inc) and Soilmaster™ DNA extraction kit (Epicentre) in accordance to the Manufacturer's instructions.

Organic Extraction using Phenol/Chloroform

Samples (total wet volume of 1 ml) in 1.5 ml microfuge tube were centrifuged for 1 min at 15 600 rcf, supernatant was removed and placed in new tube approximately 0.5 ml of premade phenol/chloroform was added (to fill tube), vortexed and centrifuged for 4 minutes at 16 100 rcf. The top layer was carefully pipetted off, as to not disturb the middle protein layer at the interface. Removed layer was added to the same volume again of phenol/chloroform vortexed and centrifuged again for 5 minutes. The procedure was repeated as often as required depending primarily on the colour of the top layer (which is indicative of contaminating substances). Final step involved the removal of the top layer and the addition of chloroform. Tubes were vortexed and centrifuged again for 2 minutes at full speed. The top layer was then added to the prepared 2 ml Centricon™ 30 000 Da concentrator, topped up with sterile molecular grade water and spun for 30 minutes at 1500 rcf at 20^{o}C. The flow through was discarded that was followed by a wash step that involved the run through of sterile water and spun again in the centrifuge. The Funnel

was then inverted in the tube and 100 µl of water is added to the inverted cap which was spun for 30 seconds at 4000 rpm. The discard was collected and kept in storage for further use.

4.4.4 Assessment of Ancient Sample Contamination by Modern Human DNA

To preliminarily evaluate contamination and aDNA quality, mitochondrial HV 2 primers L29 and H408 (Table 4.7) were used to produce a 424 bp amplicon. Using a simple PCR protocol 2 µl of sample DNA and 10 µM of each primer were added to premade *Taq* master mix (ReddyTaq, ABgene) according to Manufacturer's specifications to a final volume of 25 µl. Controls and selected samples were run periodically to check for amplicon contamination of laboratory equipment, PCR consumables and aDNA samples. PCR programme was 95 ^{o}C for 5 min; 32-38 cycles of 95 ^{o}C for 30 sec, 54 ^{o}C for 45 sec, 72 ^{o}C for 45 sec; and 1 final cycle of 72 ^{o}C for 7 min. The thermocycler used was the AB GeneAmp PCR system 2700 which for the standard for all procedures unless otherwise stated. This amplification protocol was used very rarely, in order to prevent amplicon concentration and contamination of pre and Post-PCR laboratories and predominately used for contamination & amplification rate assessment.

Table 4.7 List of Primers used in Chapter IV Including rCRS Position (where appropriate), Primer Sequences &Annealing Temperature

Primer	rCRS position	Sequence	Tm[1]
RAPD Primer 1	N/A	5'-d[GGTGCGGGAA]-3'	34
RAPD Primer 2	N/A	5'-d[GTTTCGCTCC]-3'	32
PAPD Primer 3	N/A	5'-d[GTAGACCCGT]-3'	32
RAPD Primer 4	N/A	5'-d[AAGAGCCCGT]-3'	32
RAPD Primer 5	N/A	5'-d[AACGCGCAAC]-3'	32
RAPD Primer 6	N/A	5'-d[CCCGTCAGCA]-3'	34
L29	8>29	5'-GGT CTA TCA CCC TAT TAA CCA C-3'	60
H408	410>431	5'-CTG TTA AAA GTG CAT ACC GCC A-3'	60
H16233[a]	16213>16236	5'-ACA GCA ATC AAC CCT CAA CTA TCA-3'	54
L16317[a]	16320>16343	5'-TGT GCT ATG TAC GGT AAA TGG CTT-3'	54
H16048 [a]	16030>16050	5'-TTC ATG GGG AAG CAG ATT TGG-3'	52
L16173 [a]	16175>16198	5'-ATG GGG AGG GGG TTT TGA TGT GG-3'	59
H149 [a]	128>149	5'-CTG TCT TTG ATT CCT GCC TCA T-3'	53
L323 [a]	325>346	5'-AGA TGT GTT TAA GTG CTG TGG C-3'	53
A1F [a]	15979>15998	5'-CAC CAT TAG CAC CCA AGC T-3'	57
A1R [a]	16072>16091	5'-CCC ATC AAC AAC CGT ATG T-3'	55
1F[b]	16037>16057	5'-GAA GCA GAT TTG GGT ACC AC-3'	58
1R[b]	16113>16133	5'-CAC CAT GAA TAT TGT ACG G-3'	53
2F[b]	16142>16162	5'-ATC TTG ACC ACC TGT AGT AC-3'	56
2R[b]	16220>16240	5'-CAA CCC TCA ACT ATC ACA CA-3'	50
3F[b]	16191>16211	5'-CCC CAT GCT TAC AAG CAA GT-3'	52
3R[b]	16270>16290	5'-CAC TAG GAT ACC AAC AAA CC-3'	56
4F[b]	16305>16325	5'-GTA CAT AGT ACA TAA AGC CA-3'	52
4R[b]	16381>16401	5'-CCT CAG ATA GGG GTC CCT TG-3'	63
5F[b]	16330>16350	5'-CGT ACA TAG CAC ATT ACA GT-3'	54
5R[b]	16403>16423	5'-CAC CAT CCT CCG TGA AAT CA-3'	58

[a]Gerstenberger et al., 2002; [b]Alonso et al., 2003, Hernandez et al., 2003; [1]Basic Salt Adjusted Oligonucleotide Melting Temperature (Tm) calculated using Oligonucleotide Properties Calculator (www.basic.nwu.edu/biotools/oligocalc.html)

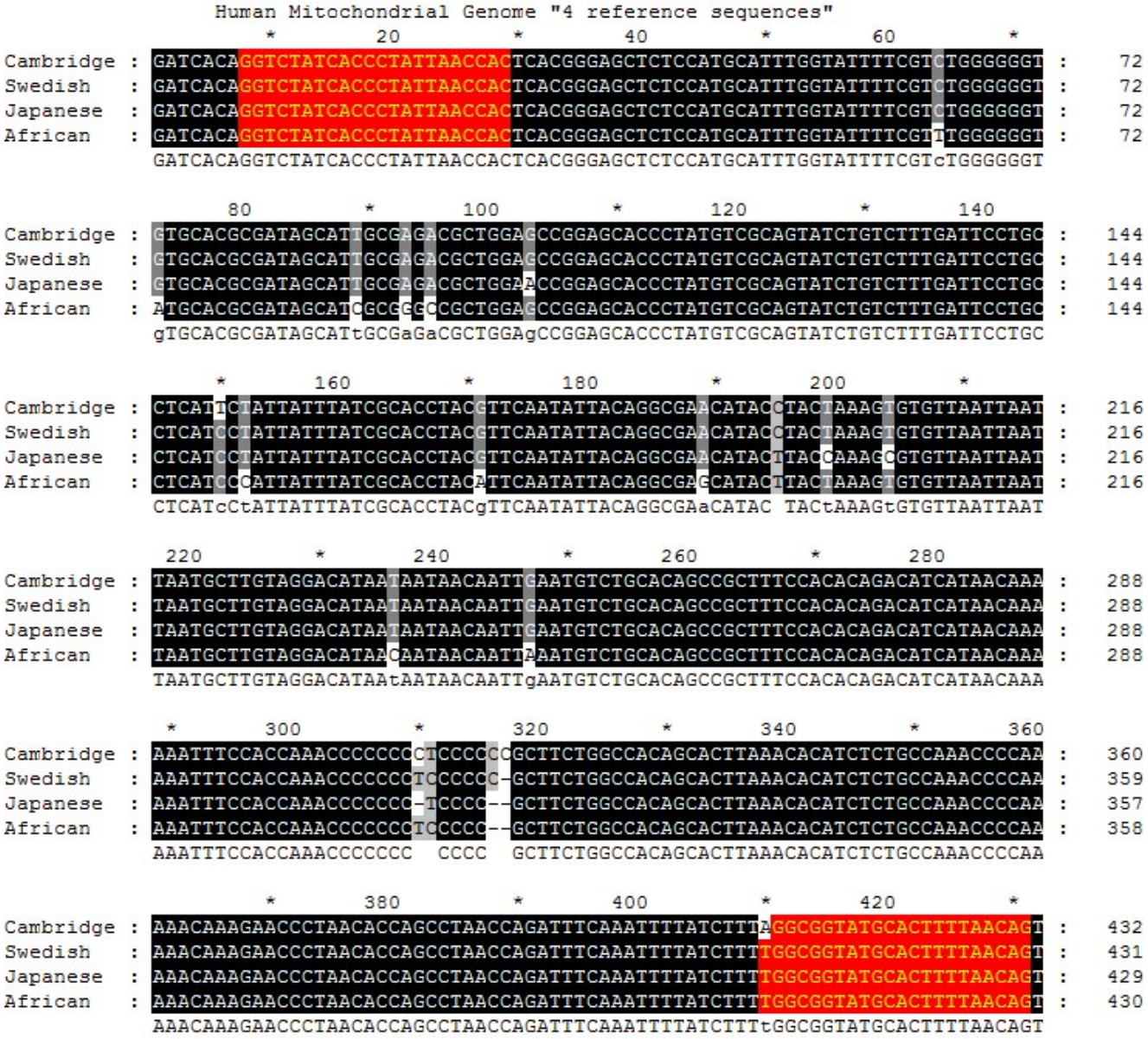


Figure 4.1: Primer Set L-29/H408 Relative to rCRS Position on the Alignments of 4 Reference Mitochondrial Sequences. The mtDNA multiplex primers reference locations are based on the D-loop hypervariable region 2 designated HV 2 (0-576 bp mtDNA position rCRS). Alignments constructed using GeneDoc, version 2.6.002 (Nickolas *et al.*, 1997), against reference sequences rCRS (NCBI reference NC001807), Swedish (NCBI reference X93334.1), African (Uganda) (NCBI reference D38112.1) and Japanese (NCBI reference AB055387).

4.4.5 Polymorphic DNA Amplification of Nuclear Sequences to Determine Species of Unknown aDNA Samples: A Developmental Study

Prior research into the forensic use of RAPD in determining the unknown animal species using the RAPD system led to an interest in looking into the identification of the many faunal fragments found at the site. This was done to assist in identification of faunal remains, to assess the variation within the samples (compared to modern controls) and also as a manner to optimise the aDNA extraction procedure itself. Comparison of the purification technique of extraction was accomplished using QIAmp mini blood extraction (Qiagen) and a simplified Chelex protocol. Chelex is a commonly used in forensics to reduce downstream processing and sample contamination. Samples A0, A2 A7, B3, B4, B5 (S1 & S2), B7 and C0 were analysed using the RAPD kit. Control Species consisted of Chelex extracted bone DNA from the following species; *Suis scrofa, Bos bublis, Ovis aries, Ornyctologus cuniculus, Canis familaris* & *Capredus caprolus*.

The RAPD Ready-To-Go Kit (Amersham Biosciences) included Ready-To-Go analysis beads, to minimize RAPD variation between reactions. Samples were processed according to the Manufacturer's instructions with some modifications. 6 different primers were used (Table 4.7) to access the samples. A value of 5 µl of DNA extract was added to a mixture of 25 µl of PCR grade water, 5 µl of a single RAPD primer and RAPD analysis bead in a 0.2 ml thin walled PCR tube. Samples placed in a thermocycler were subjected to the following PCR program: 95°C for 5 min; 45 cycles of 95°C for 1 min, 36°C for 1 min, 72°C for 2 min.

Following the results of the investigation into unknown animals, a variety of unknown human samples were investigated in order to look at any possible species or subspecies nuclear genetic markers. Using all six RAPD primers on control DNA, the effect of DNA template on the RAPD amplification was investigated using a human DNA control of known concentration and dilutions ranging from 1 ρg to 0.01 fg . Subsequently, selected samples of human bone and tooth samples from the site were amplified with the RAPD in order to observe or detect any continuity or profile resemblance between the samples.

4.4.6 Mitochondrial DNA Specific Amplification using the Gerstenberger Set of Multiplex primers.

The selected primers were specifically chosen from the literature as specific for ancient DNA from a variety of resources and as verified by data mining were robust to both contamination and unknown haplotype amplification. As a series of overlapped sequences, it was decided that this set of primers, either as an efficient multiplex, or a specific singleplex was ideal for aDNA investigations using the primer sequences as given in table 4.7. The basic thermocycler conditions the PCR reaction was optimised using a varying degree of volumes, *Taq* concentrations, annealing temperatures, $MgCl_2$ and primer concentrations, both as a singleplex reactions and as a combined multiplex, alongside with the addition of additives to improve reaction dynamics.

A multiplex mitochondrial DNA specific PCR was used to screen the samples for DNA integrity using the simple PCR protocol described below (*Taq* master mix (Reddytaq or Amplitaq Gold® + GeneAmp® Gold Buffer mix). A volume of 1-2 µl of sample DNA (template) was added to a premade mastermix containing 10 X GeneAmp® Gold Buffer mix, 1 U Amplitaq Gold, 10 mM dNTP's, 50 mM $MgCl_2$ and 10 µM of each primer made up to a final volume of 25 µl. The primers were specific for HV 1 and HV 2 areas of mtDNA as shown in figures 4.1 and 4.2. The basic PCR programme was 95^{o}C for 11 min; 38 cycles of 95^{o}C for 1 min, 56^{o}C for 1 min, 72^{o}C for 1 min; final cycle of 72^{o}C for 5 min.

The multiplex mtDNA protocol was also performed using another master mix, the Failsafe™ PCR system (Epicentre) with PCR enhancers and a range of $MgCl_2$ concentrations to further optimise the PCR reaction in the presence of inhibitors. The procedure for optimisation was according to Manufacturer's recommendation. In short, 5 µl of each Failsafe PCR 2x Premix was added to each 0.2 ml thin walled PCR tube, followed by 2.4 µl of primer mix and 0.5 µl of Failsafe PCR Enzyme Mix (1.25 Units). The cycling profile was performed as before. Once the temperature profile and influence of PCR additives was optimised, a further set of reactions were run using Amplitaq Gold and 10 X GeneAmp Gold Buffer mix with 1.5 mM $MgCl_2$ using 10 -25 µl as a final volume and normal concentration of 20 µM of each primer per reaction. This kit was used for all further amplifications.

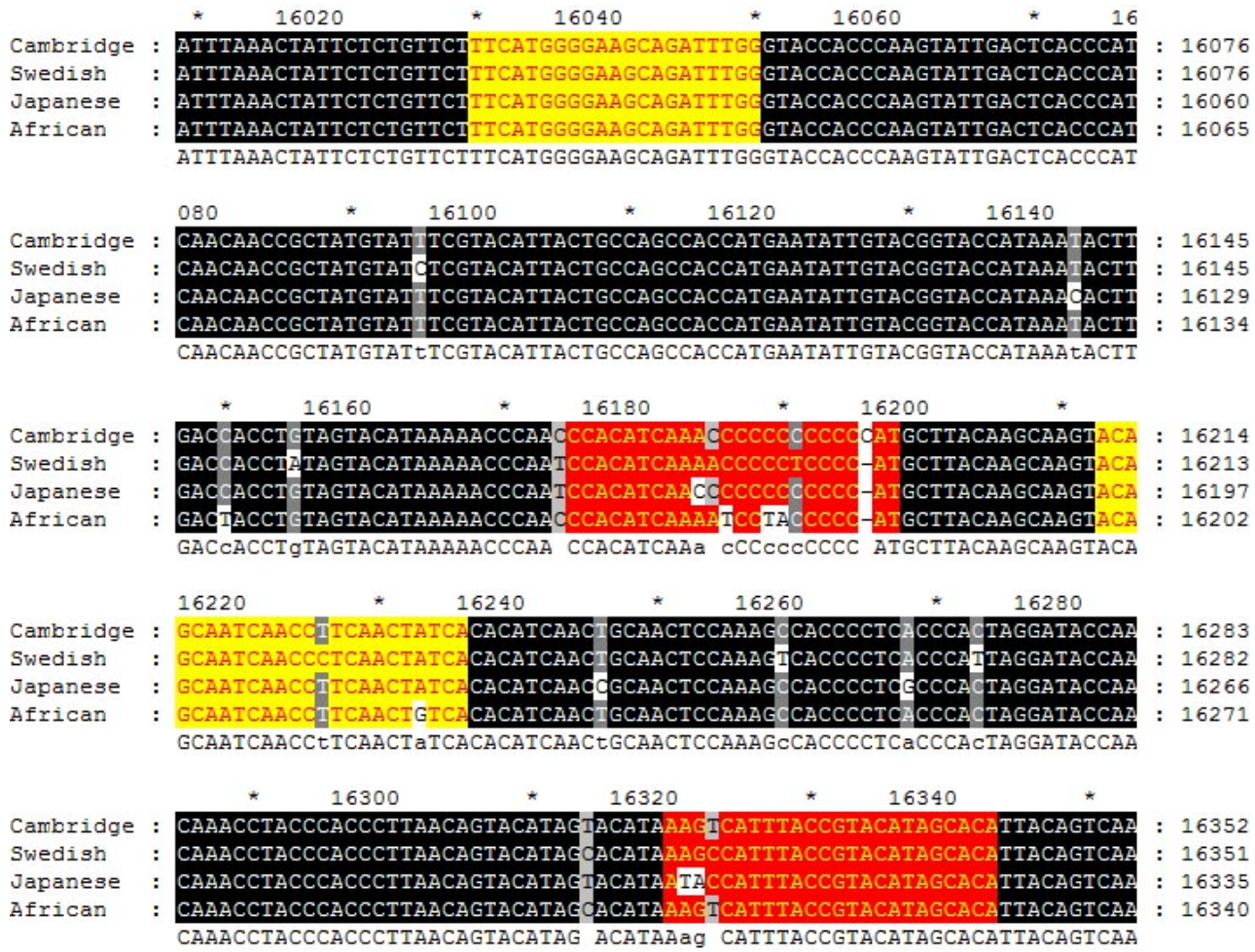


Figure 4.2: Gerstenberger Primers Sets H16233/L16317 & H16048/L16173 Relative to rCRS Positions on Alignments of 4 Reference Mitochondrial Sequences. Different primer sets are designated with different text/background combinations, H16048/L16173, red on yellow and H16233/L16317, yellow on red. The mtDNA multiplex primers reference locations are based on the D-loop hypervariable region HV1 (16024-16570 bp mtDNA rCRS position) Alignments constructed using GeneDoc, version 2.6.002 (Nickolas *et al.*, 1997), against reference sequences rCRS (NCBI reference NC001807), Swedish (NCBI reference X93334.1), African (Uganda) (NCBI reference D38112.1) and Japanese (NCBI reference AB055387).

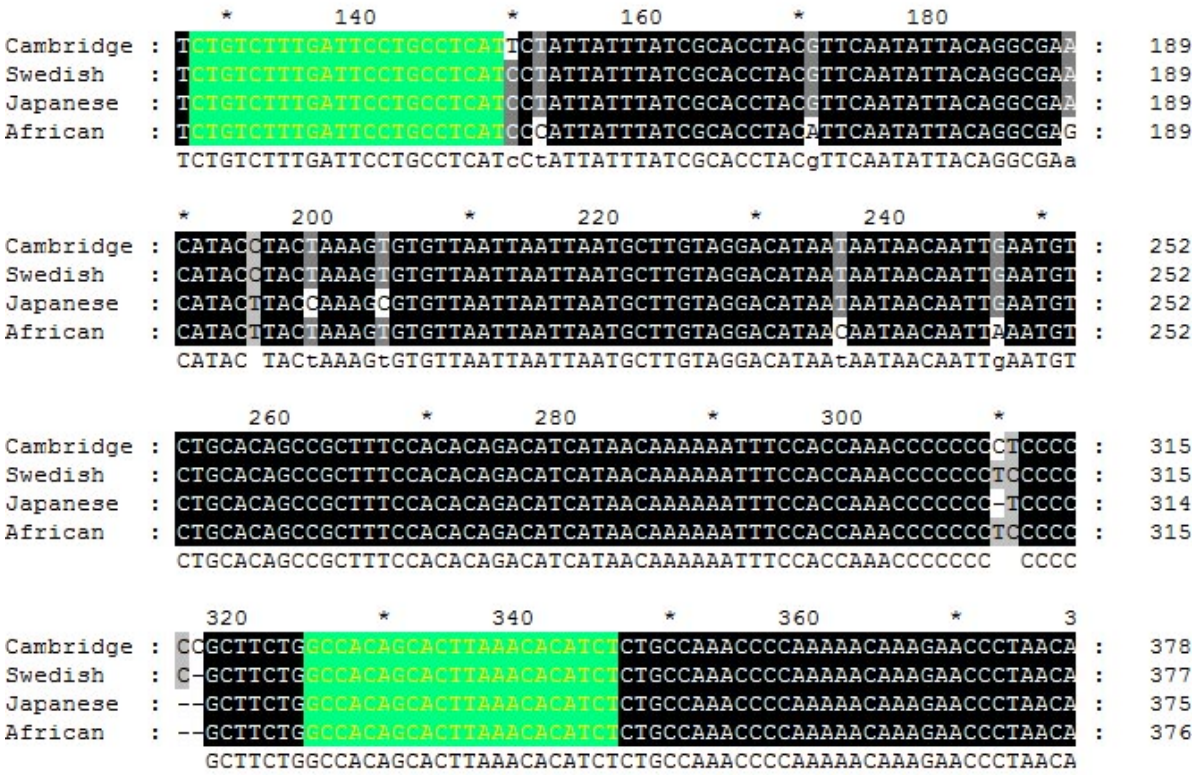


Figure 4.3: Gerstenberger Primer set H149 & L323 Positions on Alignments of 4 Reference Mitochondrial Sequences. The mtDNA multiplex primers reference locations are based on the D-loop hypervariable region 2 designated HV 2 (0-576 bp mtDNA rCRS position). Alignments constructed using GeneDoc, version 2.6.002 (Nickolas *et al.*, 1997), against reference sequences as in figure 4.1.

Ancient DNA Spiking Protocol

In order to evaluate whether or not inhibition is occurring, 3 different dilutions (10 ρg, 1ρg and 100fg) of a known concentration of the modern human control was added to the extracted DNA from ancient faunal samples A1, A2, A3 and A10 using the Gerstenberger multiplex. This was done in order to see if inhibition of genetic amplification is occurring and whether further purification or an alternative amplification protocol should be considered.

4.4.7 Mitochondrial DNA Specific Amplification using the Alonso Set of Multiplex primers.

A second set of multiplex primers (Table 4.7) referred to as the Alonso set, which all together as two multiplexes would amplify the HV1 region completely, were optimised as pairs in a singleplex and together as a 2 multiplexes for the amplification of a standard positive control before being used for the amplification of ancient tissues, predominantly the teeth. Amplitaq Gold and master mix at 1.5 mM of $MgCl_2$ was exclusively used for all Alonso singleplexes and multiplex reactions. Volumes of 1-5 µl of template were added to 1 µl of each primer (20 mM concentration) to 25 µl of master mix. The volume was made up to 50 µl through the addition of water. For singleplex reactions proportion concentrations were used but made up in a total of 25 µl. The thermocycler programme was: 1 cycle of 95^{o}C for 10 min; 36 cycles of 95 ^{o}C for 10 sec, either 60 ^{o}C (Multiplex 1: A1F/A1R; 2F/2R; 4F/4R) or 58^{o}C (Multiplex 2: 1F/1R; 3F/3R; 5F/5R) for 30 sec, 72 ^{o}C for 30 sec; final cycle of 72 ^{o}C for 10 min.

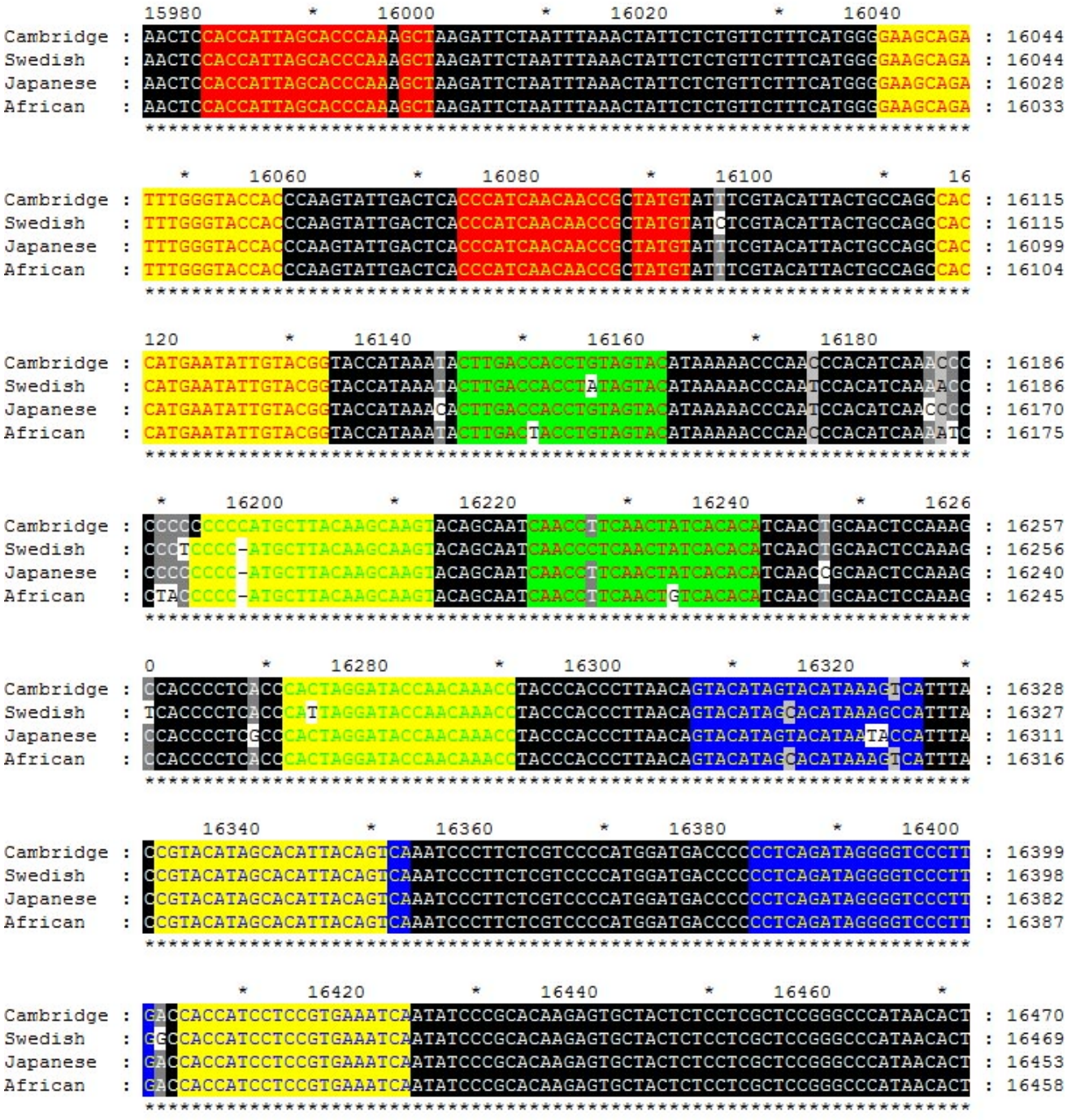


Figure 4.4: Alonso Primers Position on HV1 Alignments of 4 Reference Mitochondrial Sequences. Primer pairs are designated by background colour/text as HV1-A1 (yellow on red), HV1-1(red on yellow), HV1-2 (yellow on green), HV1-3 (green on yellow) HV1-4 (blue on yellow) HV1-5 (yellow on blue). Alignments constructed using GeneDoc, version 2.6.002 (Nickolas *et al.*, 1997), against reference sequences as in Figure 4.1.

4.4.8 DNA Detection Prior to Amplification

DNA extraction solutions were examined for the presence of DNA by thin-layer agarose gel electrophoresis on a specific a DNA horizontal gel bed and an ultra thin layer >2 mm of 0.7%, 1.5% and/or 2% Tris Borate EDTA Buffer (TBE).

Spectroscopic DNA Content Determination

DNA concentration of selected ancient and modern samples was performed using a Jenway Genova UV/Vis Biological spectrometer with a specific 200 µl cuvette, using the DNA detection 240/280 nm settings against modern control DNA 1 µg/ml.

Double stranded DNA Specific Fluorescence Spectroscopy

Selected modern and ancient samples were analysed along with some trial demineralization rate assay material (§4.3.2) in order to determine the low level DNA quantity using a double stranded DNA specific fluorescent Dye (Quant-iT™ Picogreen, Invitrogen) and a fluorescent microtitre plate reader. This technique allowed for the detection of low levels of DNA as low as 25 ρg/ml. Standard solutions of 250 µl were prepared according to the Manufacturer's instructions and analysed using a Toucan Genius Pro Fluorescent Microtitre Plate Reader at 485/535 nm (GENios Pro, Firmware: V 2.30 01/04 GeniosPRO; software: XFLUOR4GENIOSPRO Version: V 4.53).

Human DNA Specific Slot Blot Hybridization

Selected modern and ancient samples were analysed using quantification of ancient DNA. This was undertaken using Quantiblot® human DNA quantitation kit (Applied Biosystems) in order to determine if low levels of expected human DNA could be detected using the slot blot hybridization technique. Samples were processed according to Manufacturer's instructions.

4.4.9 Post Amplification DNA detection

Although more complicated than pre-amplification, due to possible issues with contamination, inhibition or even inefficient amplification, post PCR DNA detection would provide not only more reliable and specific information, but could also lead to the determination of particular information such as the genetic sequence itself.

Horizontal Gel Electrophoresis

After amplification bands were normally visualized on a 2% agarose gel using 1X TBE buffer. A volume of 1 µl of 6X tracking buffer (20% sucrose and bromophenol blue) was added to 5 µl of the amplified sample and loaded onto the gel. Samples were electrophoresed at 5V/cm for approximately 1.5 hours or until good separation of bands were observed and the leading edge of the tracking dye was 2.5 cm or less from the bottom of the gel (although conditions and measurements varied depending on the specific horizontal gel bed used). Gels were stained using a 1.0 µg/ml ethidium bromide (EtBr) bath for 20 min and de-stained in ddH_2O for another 20 min before being visualized and photographed under UV light. This was done in a specifically designed low light box that allowed for extended exposure to detect low levels of DNA (UVP BioDoc It UV Transilluminator Imaging system). DNA sizes were estimated by comparison against a molecular weight ladder, either lambda HindIII or 100 base pair ladder as appropriate.

Capillary Gel Electrophoresis

Specific highly sensitive detection and analysis of DNA fragments and bases could be achieved with the modern capillary gel techniques, with regard to this research in particular the use of the ABI 310 and ABI 3100 Automated Genetic Analyzers.

Fragment Detection

PCR products amplified with specific fluorescent primers could be analysed directly using either the ABI 310 or ABI 3100 Genetic Analyzers. A volume of ~0.25 µl of the amplification product was added to 12.5 µl of a master mix solution (12 µl of formamide and 0.5 µl of the ROX internal standard) and briefly vortexed and centrifuge in order to maximise mixing, sample was thereafter denatured by placing in a thermocycler for 3 minutes at 95^{o}C. Thereafter sample was available for analysis using the ABI310 and analysed using the ABI Genescan software.

4.4.10 Data Mining

The copious data commonly associated with genetic analysis, large databases, search protocols and complex calculations required the assistance of specific programmes and technologies in order to ascertain the appropriate information from the resulting data, these included everything from primer design to sequence analysis software.

Primer Selection and Verification

Primers were selected according to whether or not they were verifiably used in the literature and met with conditions of the theoretical estimation and matching of the oligonucleotide properties accomplished through the use of the proprietary programme Oligonucleotides Properties Calculator[1]. Homology was analysed through comparison with multiple representative entire human mtDNA sequences compiled in GeneDoc (Multiple sequence alignment Editor & Shading Utility v2.6.002; Nickolas et al., 1997). The specificity was checked through direct searches through the BLASTn interface of the both the DNA databases of the National Center for Biotechnology Information (NCBI)[2] and the DNA Databank of Japan (DDBJ)[3].

Electrophoretic Gel Analysis

Electrophoresis gels, particularly the complex RAPD gels were analysed using proprietary software such as TotalLab version 2003.03 1D gel analysis. This involved the measurement UV intensity of the EtBr stained DNA band according to peak height and molecular weight (calibrated against a 100 bp ladder). This allowed the estimation of the molecular weight of each band and an exact molecular weight fingerprint to be formed for each sample. For comparative purposed this also gave an approximate concentration of the DNA in the fluorescent band (compared to the control ladder).

Statistical Analysis of Data

Quantitative data are presented as mean ±SE on the appropriate experimental graph. Where appropriate trend lines are formulated mathematically. Where populations are a considered paired student's *t*-tests are performed, and a value of $\rho=0.05$ taken as significant. Due to the nature of ancient DNA research (hit or miss) and the qualitative aspect of DNA analysis (either a positive or negative result) it is often not possible or appropriate to perform statistical analysis. aDNA. Results are shown where available and the qualitative research shown is representative.

[1] www.basic.nwu.edu/biotools/oligocalc.html
[2] www.ncbi.nlm.nih.gov/
[3] www.ddbj.nig.ac.jp/

4.5 Results and Discussion

As explained previously, the study of ancient DNA samples requires consideration of the potential for contamination. The approach used in these studies concentrated on simplifying procedures to their most essential components, and minimal handling of site samples throughout experimentation in the laboratory. In addition, specific protocols were employed to maximise the extraction of DNA from the ancient samples.

4.5.1 Determination of the optimal conditions for EDTA demineralization of calcified tissues.

The first step in acquiring DNA from the sample bones or teeth was to breakdown and separate the hydroxyapatite/collagen structure of the bone. This was accomplished whilst minimising th loss and damage to the DNA contained within. From the literature (listed in Table 4.8) an effective decalcification solution contains three components: a calcium sequestering molecule, a detergent and a protein specific degradative enzyme. Ethylenediaminetetracetic acid (EDTA), a water soluble polyamino carboxylic acid, is the tool of choice with regards to calcium sequestration. It is useful in that, in addition to gently extracting calcium, it also protects the DNA from enzymatic degradation by DNAses. A detergent is also used which segregates components once separated, including fatty acids and cell structures.

Variations in specific detergents and their concentrations selected in different protocols are mostly with regards to availability and effects on the other components in the mixture, such as the activity of proteinase K. Assays of sodium dodecyl sulphate (SDS) show that, in the presence of up to 2%, proteinase K remains very active. Finally, the enzyme of ideal choice is collagenase, but since this is not compatible with EDTA, experiments involving collagenase require further steps that are experimentally sensitive to contamination.

However, brief experimentations using X-Ray radiography of preliminary decalcification (Figures 4.5 & 4.6) solutions show that conclusions garnered from current publications, regarding necessary times, temperatures and in particular volumes may be misleading.

X-ray Observations of Decalcified Bone Powder

Simple X-ray observations were carried out on decalcified solutions containing 50 mg of powdered faunal site bone in 2 ml of decalcification solution, which had been left for a period of between 1 to 5 days and at either 37^{o}C or 56^{o}C, The results in figures 4.5 and 4.6 showed a pellet of calcified material that appeared to be the same size irrespective of these conditions.

Considerations of pH with regards to the Decalcification Solution

An essential consideration with regards to both EDTA and proteinase K is that both are sensitive to changes in pH, which may affect the rate of reaction. It is also usual in protocols to maintain DNA in a solution at a pH of around 8. Therefore, many protocols use pH buffered solutions. However, at the microlitre volume level, pH does not seem to play a particular problem, apart from the necessary establishing the EDTA pH at 8.0.

A)

Figure 4.5a: (Above). X-ray radiograph of the base of 2 ml microfuge tube after centrifugation, looking at the material pellets after decalcification over a period of 1-3 days at 37 ^{o}C (left-right).

B)

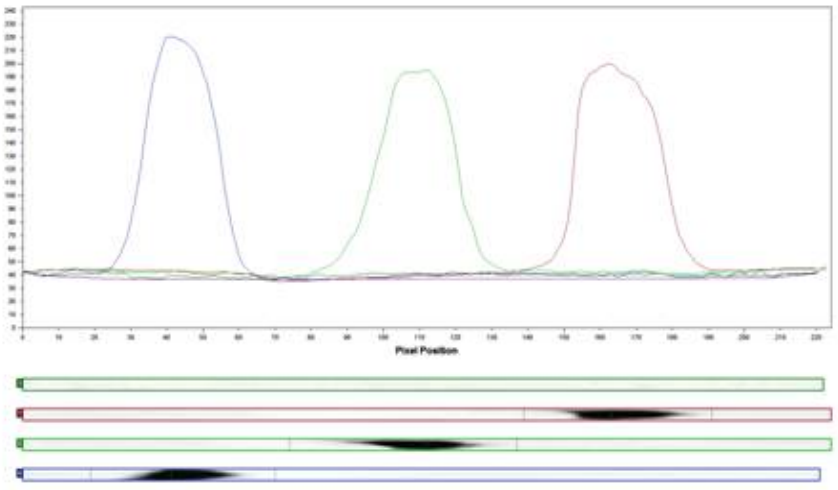


Figure 4.5b: (Above). X-ray transmittance intensity & volume for each tube in sequential order of days 1, 2 & 3 (left-right).

A)

Figure 4.6a: (Above) X-ray radiograph of the base of 2 ml microfuge tube, after centrifugation, looking at the material pellets after decalcification over a period of 1-5 days at 56°C (left-right/top-bottom).

B)

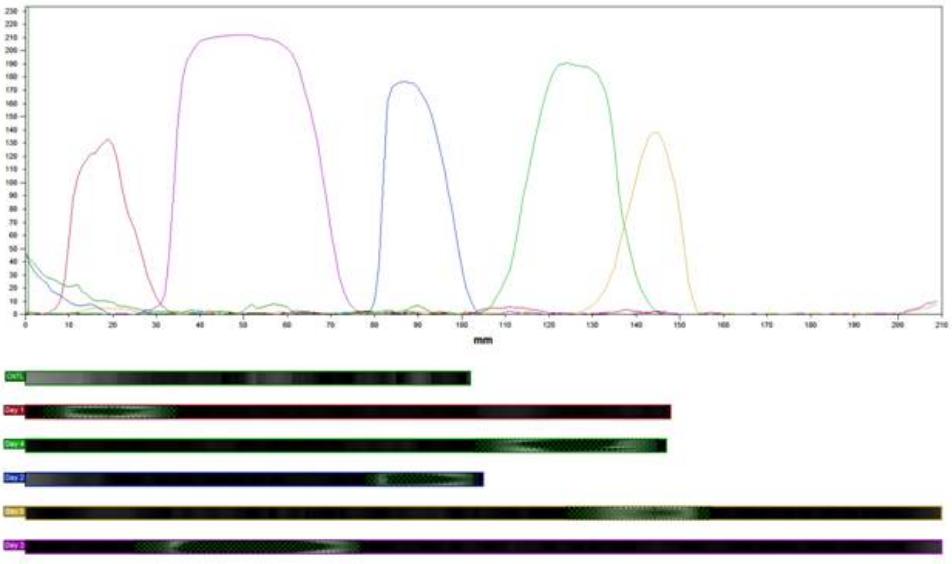


Figure 4.6b: (Above) X-ray transmittance intensity and volume for each tube in sequential order of days 1, 3, 2, 4 & 5 (left to right).

Temperature Considerations in the Decalcification Solution

An important factor in any chemical or enzymatic reaction is temperature. The optimal reaction temperature is dictated by the active ingredients, which in this case are EDTA, whose activity is mainly dictated by pH, and proteinase K. The reaction times alluded to in most of the literature, coupled with the volumes required, are misleading. According to the Qiagen proteinase K Manufacturer's information booklet, the peak activity of the proteinase K is at 65^{o}C in a simple EDTA solution. The results of the present study however showed that the optimum time is 1 hour, whereas most of the experimental decalcification in this chapter takes place overnight.

Determination of the Rate of Decalcification with EDTA

The results of the X-ray radiography of the contents of microfuge tubes made it necessary to investigate further the rate of decalcification of samples using different solutions, as it appeared that a large part of the bone or tooth remained calcified. This was important since the bone sample were not fully digested, and apparently, not all of the DNA sample would be extracted, since, as explained in chapter 1 (§1.5), DNA is intimately associated with the inorganic (or calcium) component of bone. In addition, un-degraded, or semi-degraded collagen subunits are suspected of interfering with the subsequent Polymerase Chain Reaction. Clearly, considering the scarcity and quality of aDNA in these samples, it was imperative to optimise the recovery of aDNA from the powdered material.

A series of assays were therefore conducted to determine the rate and extent of EDTA decalcification in the small volumes of samples and solutions used as microvolumes are most commonly used in aDNA laboratories. In order to monitor the calcium levels in these solutions, AAS was selected. Due to the sensitivity of this technique, and the degree of dilution required, experiments were conducted using the calcium endpoint colorimetric indicator ammonium oxalate and UV spectroscopy. Ammonium oxalate binds to calcium and forms a complex that flocculates. This can in turn be detected at the lower end of the UV/vis scale at a wavelength of around 400 nm. As shown in figure 4.7, calcium levels in solution were observed to increase over a period of 1 hour.

Table 4.8: Some Commonly used Decalcification Buffers in the Literature					
References	**Components**	**Concentrations**	**pH**	**Temp**	**Time**
Kuntzee, 1996	EDTA	0.3 M	7.8	55	12 +
	Tris-HCl	10 mM			
	Sodium Acetate	1 mM			
	SDS	1%			
	proteinase K	N/A			
Hanni, 1994	EDTA	0.5 M	8	50	18
	N-laurylsarcosine	0.10%			
	proteinase K	N/A			
Ivanov, 1995	EDTA	0.5 M	8	37	12
	Tween-20	0.50%			
	proteinase K	N/A			
Perry, 1989	EDTA	90 mM	7.8	55	2
	Tris	45 mM			
	SDS	0.50%			
	N-laurylsarcosine	0.50%			
	proteinase K	N/A			
Hoss & Paabo, 1993	EDTA	0.02M	8	60	2+
	Tris	0.1M			
	Guanadine thiocyanate	10 mM			
	Triton X-100	1.30%			
	Proteinase K	N/A			
Fattorini, 1989	Na2EDTA	10 mM	8	40	12+
	Tris	10 mM			
	NaCl	100 mM			
	Dithiothreitol	40 mM			
	SDS	1.30%			
	Proteinase K	N/A			
Gelsthorpe, 1996	Tris-HCl	0.1 M	7.8	N/D	12+
	Na2EDTA	0.50%			
	NaCl	0.1 M			
	Nonidet P40	1%			
	proteinase K	N/A			

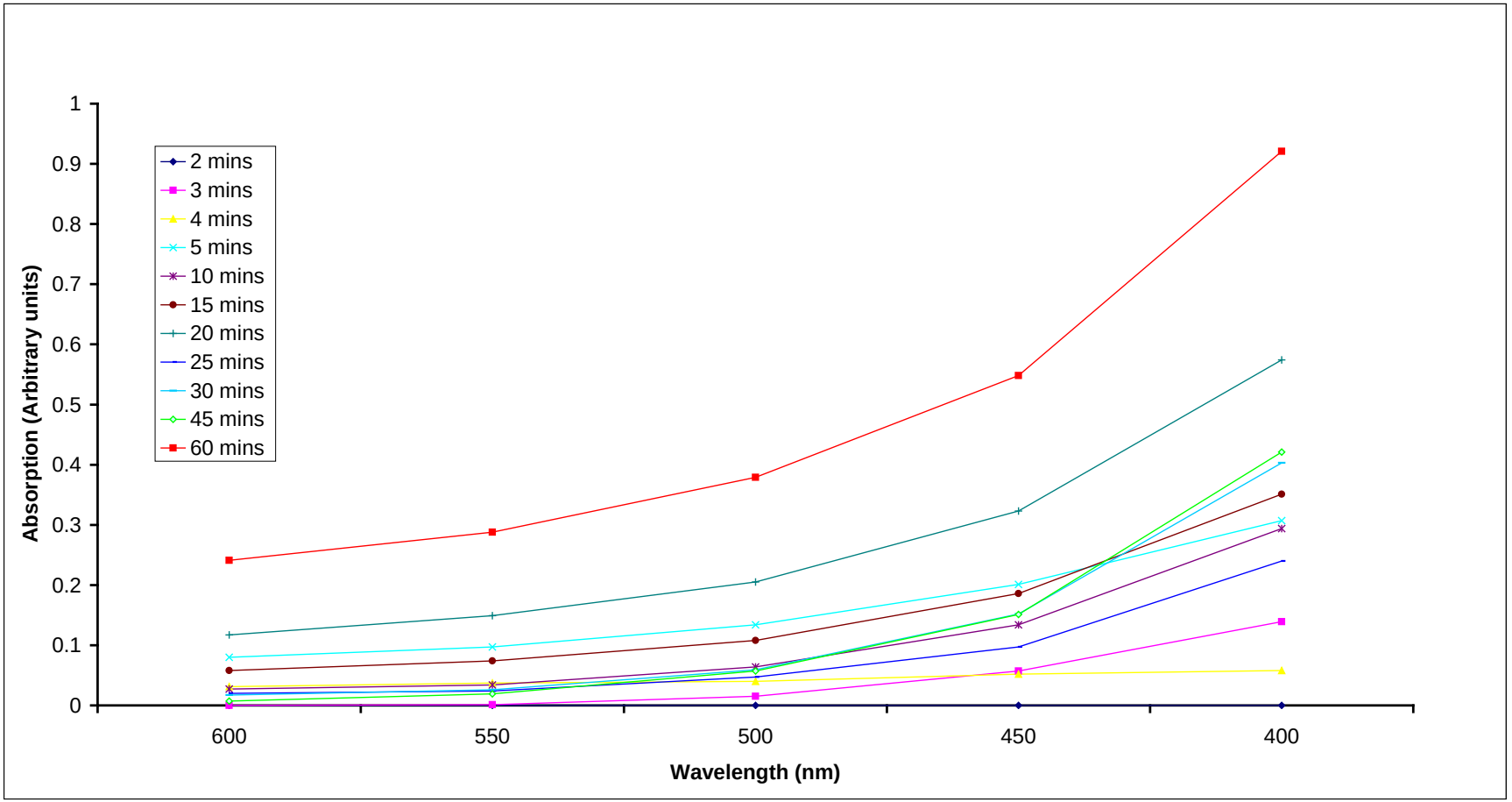


Figure 4.7: Absorbance of the EDTA Decalcification Solution after 2-60 min Incubation with Bone powder as detected by UV/vis and the addition of ammonium oxalate. Each line represents the absorbance at a particular time (ie 60 min) over a range of wavelengths (between 600 and 400 ηm). From the graph UV/vis differentiation of different levels of the calcium in solution occurs best at 400 ηm.

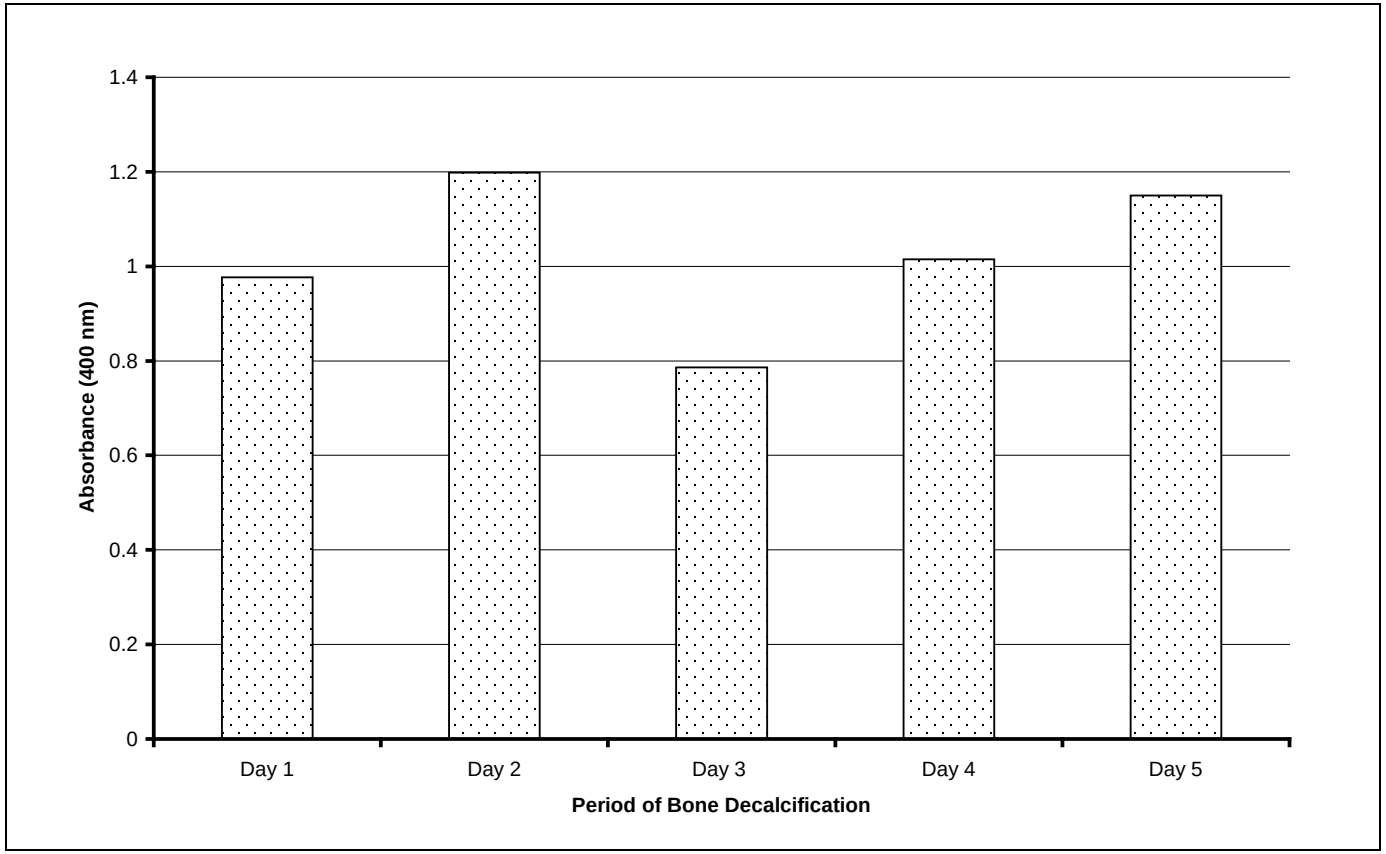


Figure 4.8: Calcium in Solution after EDTA Decalcification for 24 to 120 hours (1-5 days).
Calcium detected using the absorbance of ammonium oxalate detected by UV.vis Absorption at 400 ηm.

Rate of Reaction: Incubation Time

The above results show that decalcification of samples, as determined by colorimetric measurements (Figure 4.8), and X-ray radiography, in small volumes, reaches the end point in less than 24 hours, and that longer periods of decalcification are not necessary. Indeed, longer times could actually be detrimental as the saturated EDTA in solution may not be as efficient in sequestering the heavy metals required for the activity of some enzymes, such as DNAses. Bone decalcification, as verified by ammonium oxalate endpoint absorption assays at 400 nm, indicates that the level of calcium in solution may vary from one sample to another over time. However, once the calcium reaches a saturation point the calcium levels in solution do not increase far beyond this point, and that this occurs rather quickly.

In order to pin point the rate of reaction, further assays were performed, and the results obtained using AAS and ammonium oxalate absorbance assays are shown in figure 4.9 and figure 4.10. These results clearly show that the reaction endpoint, or rather final lag phase, occurs between 15 and 45 min, and that within one hour the reaction rates reach saturation. Surprisingly, however, the AAS results for mg/ml indicate that on a per molecule level that the reaction is only approximately 65% efficient.

Rate of Reaction: Volume Constraints

Clearly reaction volumes are important in achieving effective dissolution of all of the bone material prior to DNA extraction. Unfortunately, equipment availability, space and convenience become constraints when dealing with larger as facilities such as larger centrifuges are generally centralised, and this introduces issues regarding the potential for contamination from other users. Therefore, preference was given in this research project to small volumes. Considering the results of the previous experiments with regards to the rate of reaction, two substitutions of 15 min and 30 min were performed for between 8 and 16 substitutions in order to determine the efficiency of bone and tooth decalcification using both the AAS and ammonium oxalate absorbance (Figures 4.11 to 4.16).

Clearly, both techniques are efficient, although the teeth appear to decalcify better than bone, which is most likely a function of the density of the biological faction interfering with the decalcification reaction. In addition, the EDTA continues to be around half as efficient as it should be if it considered on a mole to mole basis. This is perhaps related to the possible influence of pH and reaction temperature. With regards to the ammonium oxalate results, bone appears to have a higher absorbance over a longer period. This may indicate either that absorbance values associated with teeth samples are very low, and hence reflect a lower efficiency of detection or that the larger organic faction of bone release molecules into solution that absorb at the same wavelength, giving falsely high results.

The broad spectrum of the AAS results over the various time periods and conditions above demonstrate the ultra-sensitivity of the AAS technique and intra sample variability within the rate. However, these results are consistent with the expected 50-65% efficiency of the EDTA chelation from the first experiments where, at the volumes used, it takes between 8-10 substitutions to fully decalcify samples (Figure 4.14). Clearly, to fully dissolve a 50 mg sample of bone or tooth, 10 ml of decalcification solution should be used, and greatest efficiency requires 5 substitutions of 2 ml. In order to fully capitalise on peak proteinase K activity, samples should be incubated at 56-65^{o}C for 1 hour, before substituting with a fresh solution.

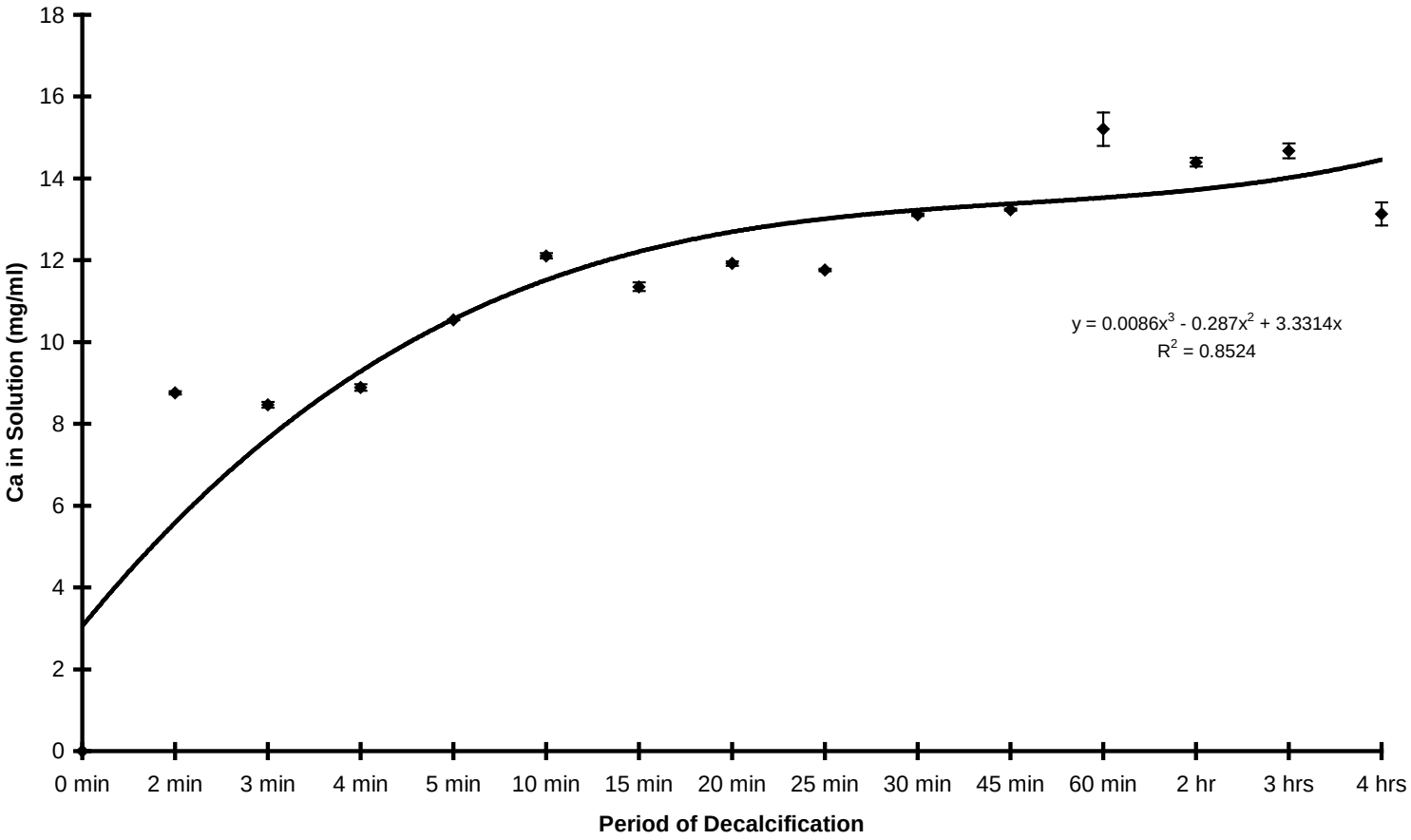


Figure 4.9: Calcium in EDTA Solution from the Decalcification of Animal Bone Powder over time as Detected by AAS. Results typical of such experiments (n=3-6).

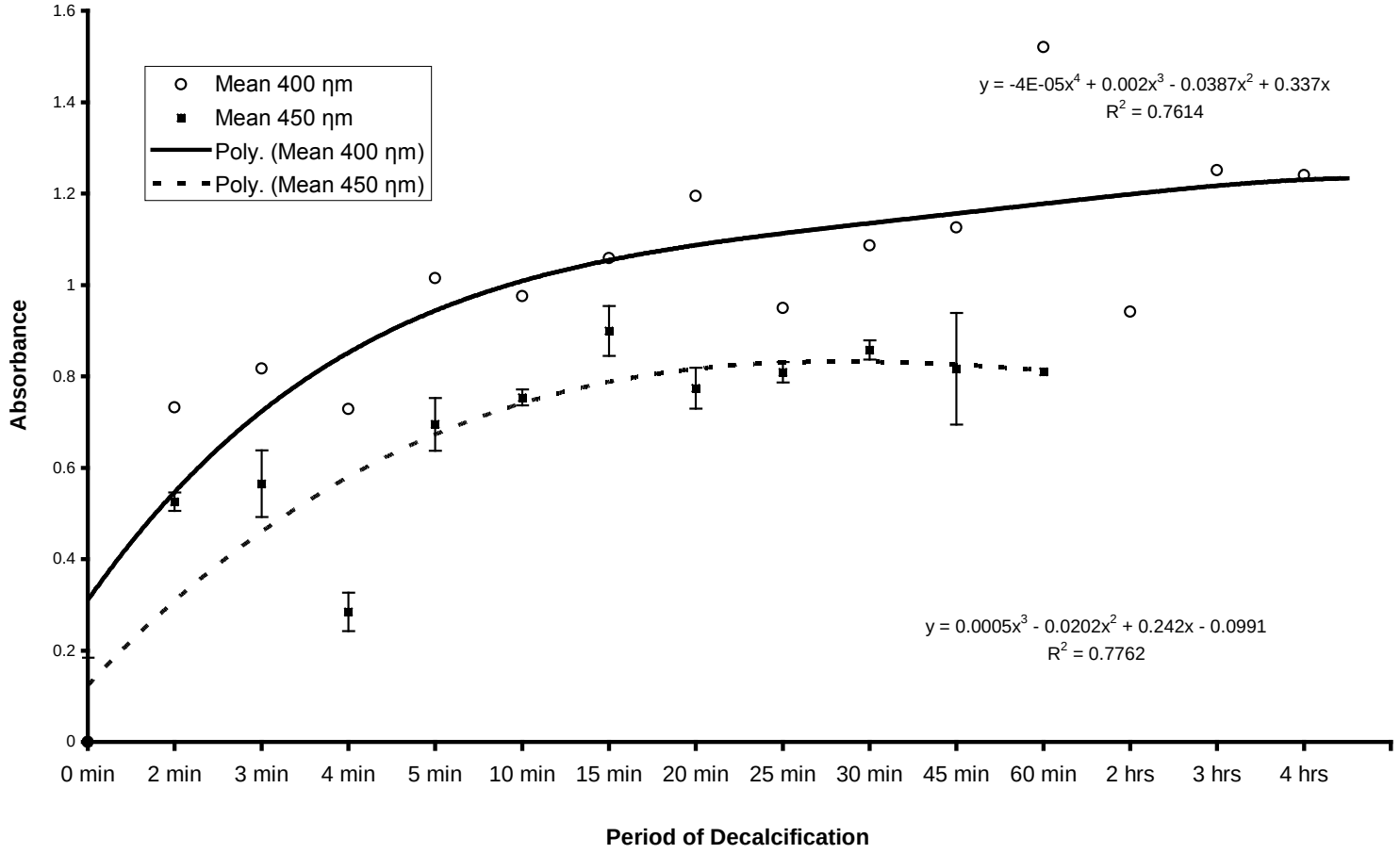


Figure 4.10: Calcium in EDTA Solution from the Decalcification Animal Bone Powder over time as Detected by the Absorbance of Ammonium Oxalate. Results typical of such experiments (n=1-3).

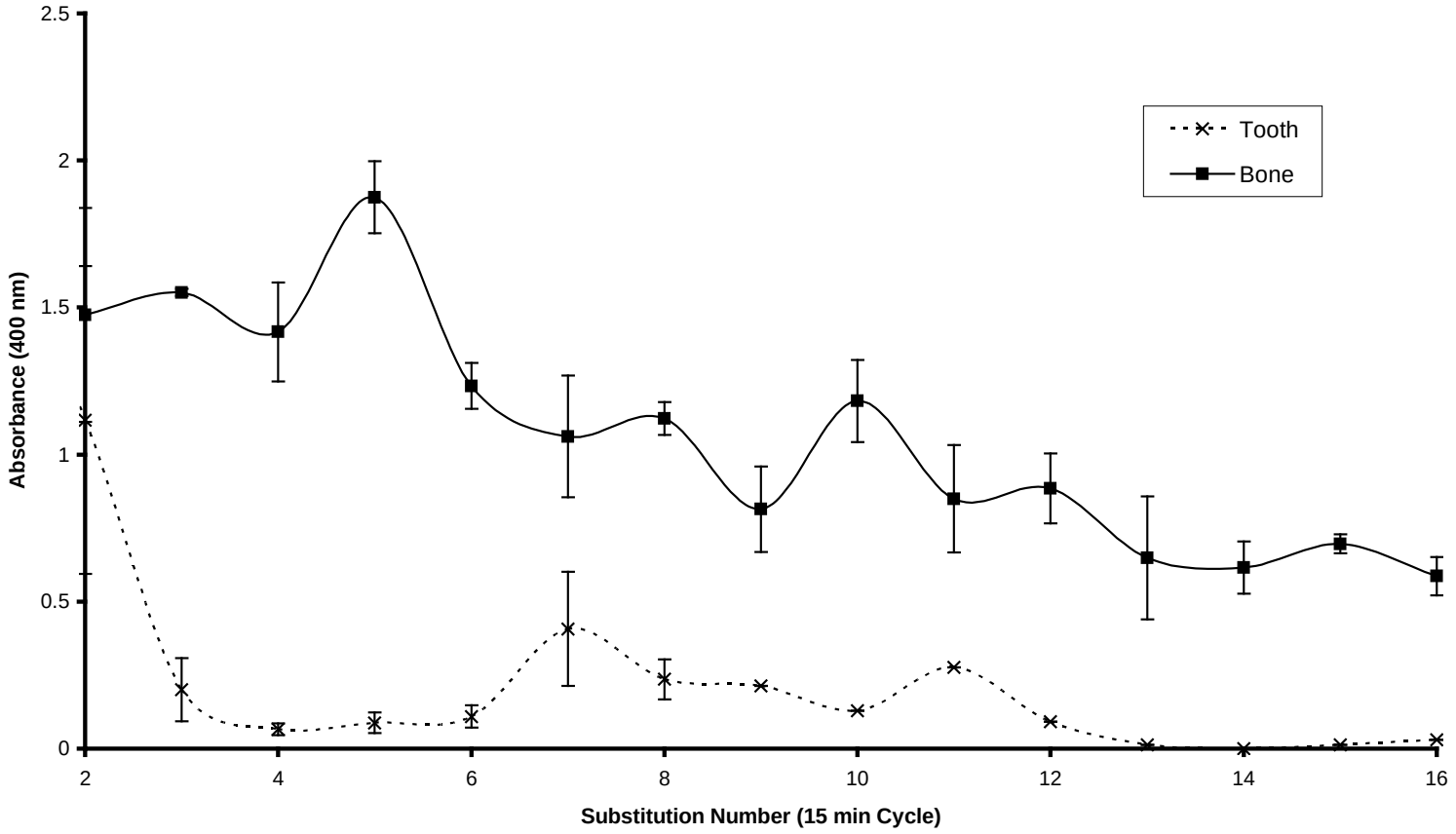


Figure 4.11: Calcium in EDTA Solution from Tooth and Bone Powder over a Number of Substitutions (15 min cycle) as Detected by the Absorbance of Ammonium Oxalate. Results typical of such experiments (n=3).

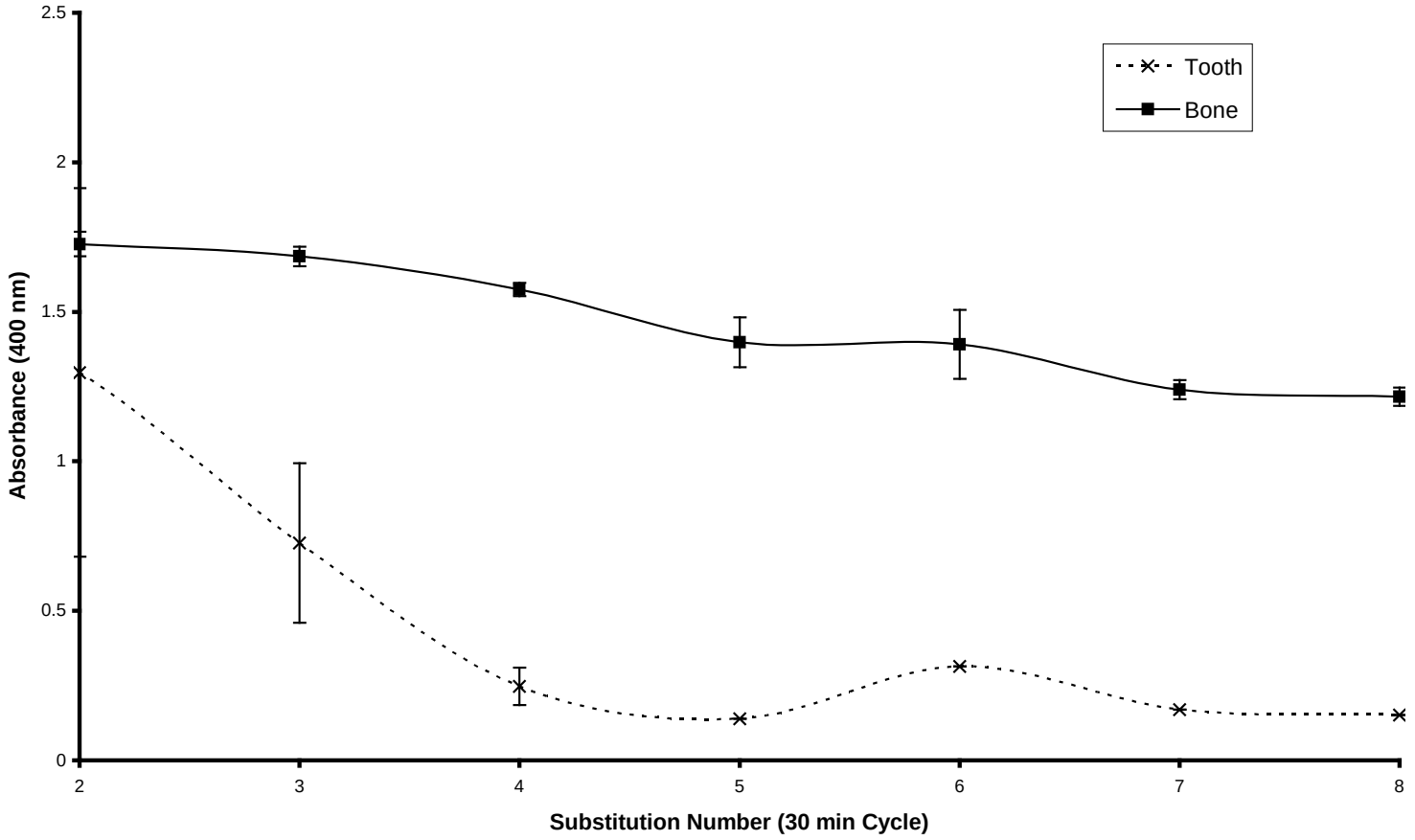


Figure 4.12: Accumulation of Calcium in EDTA Solution in Tooth and Bone Powder over a Number of Substitutions (30 min cycle) as Detected by the Absorbance of Ammonium Oxalate Results typical of such experiments (n=3).

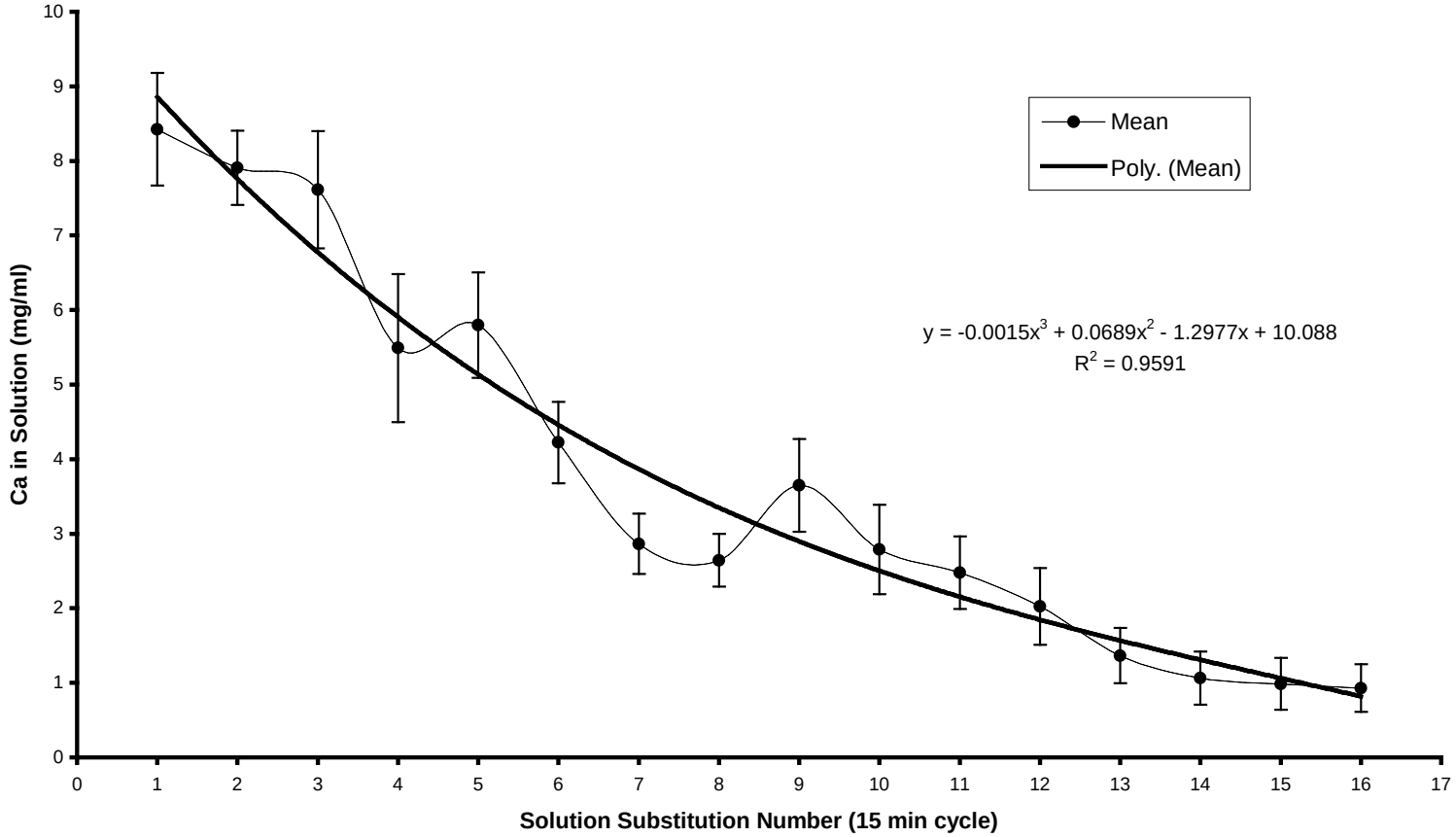


Figure 4.13: Accumulation of Calcium in EDTA Solution from Tooth Powder over a Number of Substitutions (15 min cycle) as Detected by AAS. Results typical of such experiments (n=9).

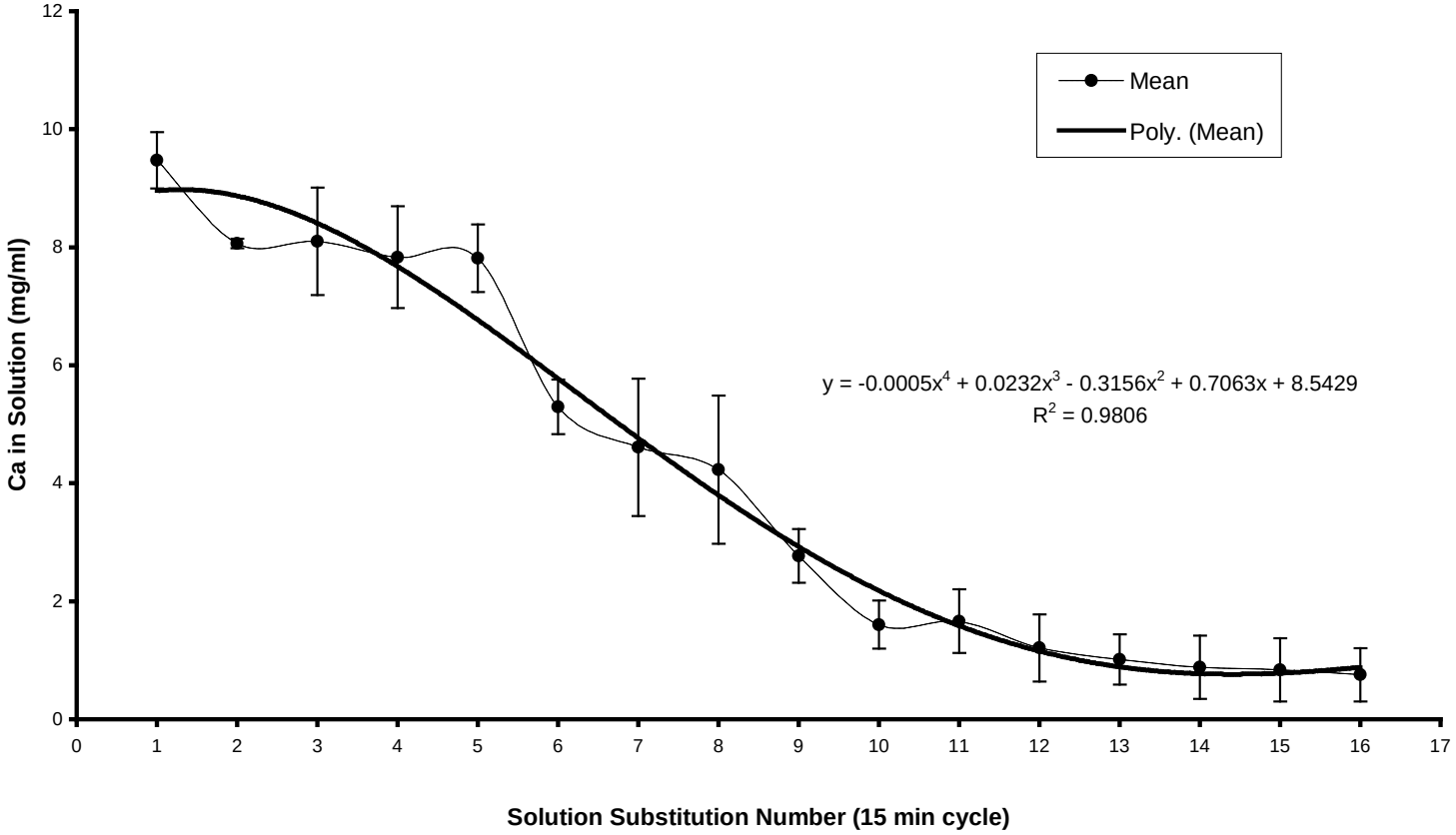


Figure 4.14: Accumulation of Calcium in EDTA Solution from Bone Powder over a Number of Substitutions (15 min cycle) as Detected by AAS. Results typical of such experiments (n=6).

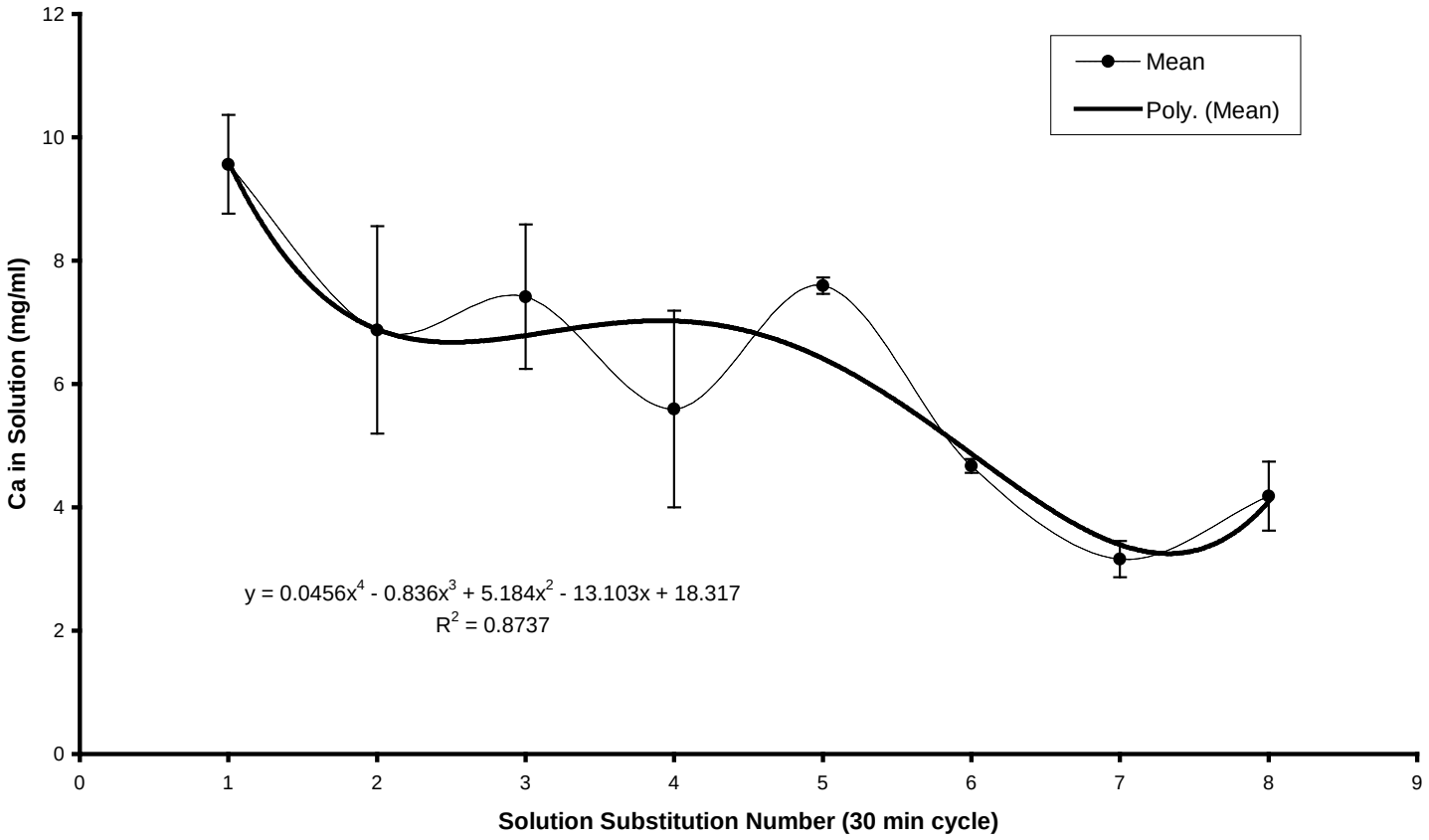


Figure 4.15: Accumulation of Calcium in Solution in Tooth over a Number of Substitutions (30 min cycle) as Detected by AAS. Results typical of such experiments (n=9).

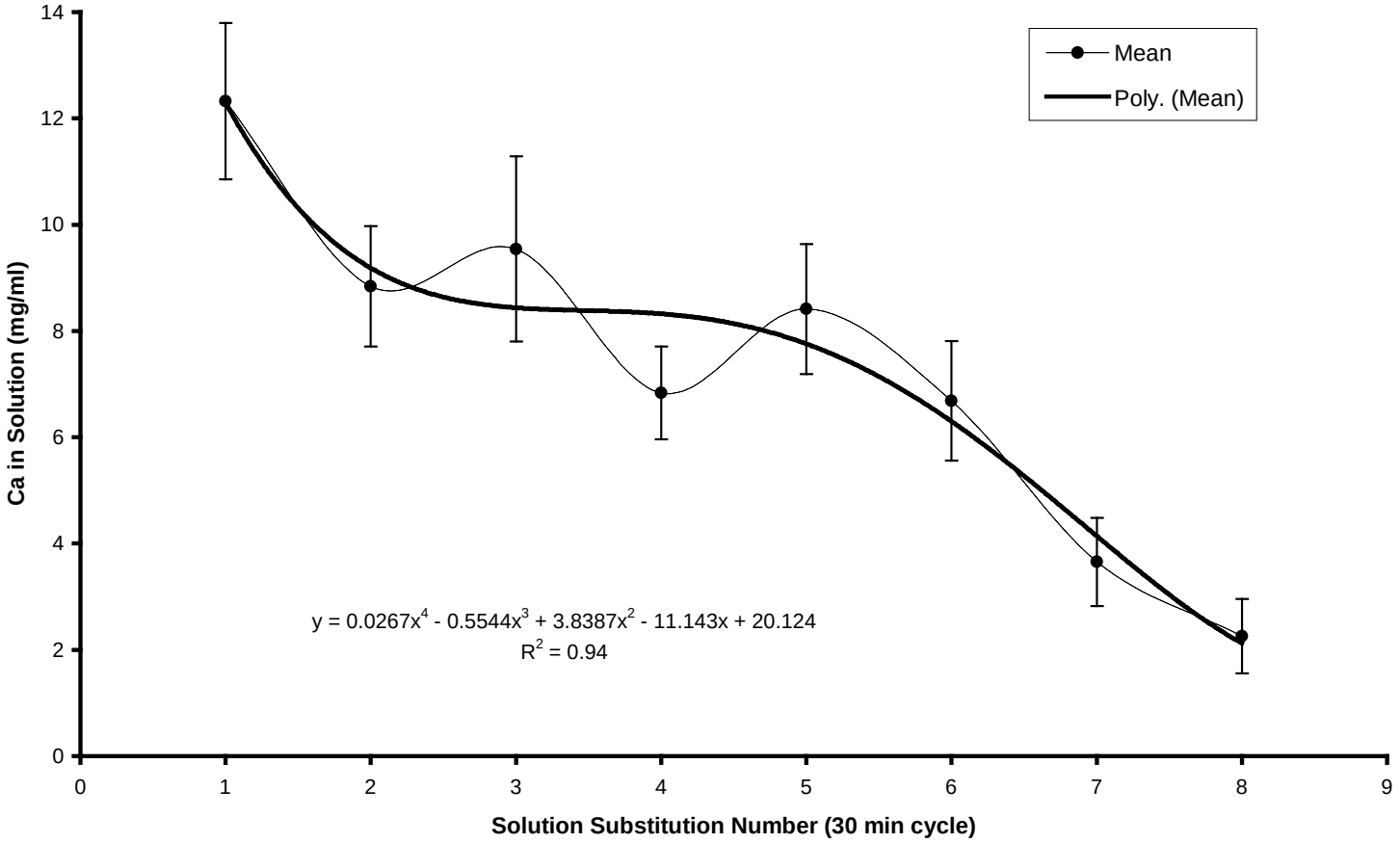


Figure 4.16: Accumulation of Calcium in Solution in Bone over a Number of Substitutions (30 min cycle) as Detected by AAS. Results typical of such experiments (n=6).

4.5.2 Extraction and Purification of aDNA from the Towyn-Y-Capel Tooth and Bone Samples using Organic and Chaotropic Techniques

A spiked sample of 100 fg of sonicated human DNA was added to 50 mg samples of powdered animal bone samples in order to compare efficiencies of different DNA extraction techniques. The methods used were extraction protocols using chaotrophic, organic or Chelex based separation techniques. Chaotrophic based extraction used guanidine thiocyanate, followed by silica purification, which is currently the most popular of the commercially available techniques. In total, 5 different kits were compared, including the aDNA specific Bio101 kit, the commonly used QIAmp mini Blood kit (commonly referred to as the Qiagen kit) and other proprietary soil and forensic extraction kits (§4.3.3).

Organic extraction, using phenol/chloroform, is a basic standard protocol in aDNA recovery, and is consistently used in most aDNA laboratories, either with or without a subsequent purification step using silica columns. Kits were favoured due to the restricted aDNA laboratory conditions available, which restricted the use and availability of a large centrifuge which would be required for phenol-chloroform extractions (as the large centrifuge was in another laboratory and was used by many other researchers).

Commercially available chaotrophic extraction kits also have the advantage of being pre-aliquoted into small segregated volumes, with known batch numbers and provenance, ready for immediate use, and should hence produce more standard and reproducible results. The kits were selected on the basis of their suitability for aDNA and trace DNA extraction, and reported use in recovery of aDNA. These included Qiagen, a common used kit in forensic applications for trace DNA samples, aDNA geneclean kit, which was the only one designed for aDNA, and Soilmaster kits, containing proprietary procedures to deal with the specific issues of humic and fulvic acid contamination of samples. Of these kits, the Geneclean aDNA kit provided the best signal and consistent quality, and was therefore selected for primary use.

Towyn-Y-Capel Skeletal Population DNA Extraction Phases

Once decalcification and extraction/purification procedures were selected and optimised, extraction of DNA from the main skeletal population samples was undertaken. During the progress of this research, five distinct extraction phases were undertaken.

These are represented by distinct periods of time where no amplification or manipulation of genetic material was undertaken in between to prevent contamination and sample cross over. No more than ten samples were handled at any one time.

Phase 1 Extraction:
Samples: Season 1 Bones and Teeth
Decalcification Protocol: 100-200 mg in 2 ml volumes of decalcification buffer
Extraction/Purification: Bio 101 aDNA kit

Phase 2 Extraction:
Samples: Bones and Teeth
Decalcification Protocol: 100-200 mg in 2 ml volumes of decalcification buffer
Extraction/Purification: Qiagen mini blood DNA kit

Phase 3 Extraction:
Samples: All teeth Samples
Decalcification Protocol: 100-200 mg in 2 ml volumes of decalcification buffer using PTB
Extraction/Purification: Bio 101 aDNA kit

Phase 4 Extraction:
Samples: All Teeth Samples
Decalcification Protocol: ~100 mg in multiple 2 ml volumes (>5) of decalcification buffer using PTB
Extraction/Purification: Bio 101 aDNA kit

Phase 5 Extraction:
Samples: All Teeth Samples (where available)
Decalcification Protocol: 100-200 mg in 40 ml volumes of decalcification buffer, concentrated using concentrator Bio Spin
Extraction/Purification: Bio 101 aDNA kit

4.5.3 Quantification of aDNA

One of the greatest issues surrounding forensic and ancient tissue genetic analysis is the ability to ascertain the genetic value of a sample without the expensive waste of resources and time on the *ad-hoc* amplification of unknown sample. In order to assist in this a number of technologies have recently been brought into the field and were used during this research. These are briefly described below.

Spectroscopic DNA Content Determination

This is a technique commonly used for measurement of DNA concentrations. Trials have shown that aDNA levels of around 1 ηg control are beyond the limits of detection using this technique due to sample sizes, despite the use of low volume micro-cuvettes. This was deemed unusable for aDNA studies.

Human DNA Specific Slot Blot Hybridization

This is a technique of *in situ* hybridization specific for human DNA and has come to the fore recently for its use in screening forensic samples. A total of 15 bone and teeth samples were processed according to these techniques, and although the high sensitivity of the technique was verified by the control DNA samples, no human DNA could be detected in the aDNA extracts. This was possibly due to the scarcity of the DNA in the extracts, but could also have been due to the presence of inhibitors. Quantiblot results (not shown) were overwhelmingly negative for the trial 15 random burial site samples, although positive results were obtained with mitochondrial sequence amplification. Considering the estimated age and preservation conditions of samples of teeth and bones used, this was not unexpected, as the limit of detection of the Quantiblot technique is considered to be approximately 0.1 ηg of DNA, below that expected of aDNA samples. This indicates that the amplification of nuclear targets of aDNA would be highly unlikely.

DNA Specific Fluorescence Spectroscopy

Recently coming into the fore as a more sensitive form of DNA detection, Quant-iT™ picogreen fluorescent microtitre plate detection uses a modified form of fluorescence detection for DNA and microtitre plates. This is becoming preferred to other techniques for DNA quantification due to its simplicity, large sample number throughput and its suitability for automation. Unfortunately, the concentration of aDNA in extracts was too low to be detected due to fluorescence crossover from positive controls and calibration ladder into surrounding microtitre plate cells. This proximity was a particular problem, despite subsequent attempts at spreading the samples apart and that meant this technology was not suitable detection of samples containing less than 1 ηg of DNA.

DNA Quantitation using Real-Time PCR

Strongly recommended by some for aDNA procedures (Gilbert et al., 2003; Kefi et al., 2003; Alonso et al., 2004), real-time PCR using the Roche Lightcycler v1.0 and SYBR fluorescent chemistry was carried out using the two primers sets from the Alonso multiplex, namely A1F/R and 5F/R due to the diminutive amplicon size. However, the amplification profile, due to the unique thermocycler ramping and amplification chemistry, was considerably different from the normal profile used, and therefore provided limited additional information in return for the time and resources required to optimise this technique for aDNA. Hence, this was not pursued further.

4.5.4 Amplification of Polymorphic nuclear DNA sequences

In 1990 Williams et al., developed a single primer based PCR technique that allows the rapid generation of polymorphic markers or Random Amplified Polymorphic DNA (RAPD). The reaction is carried out under low stringency conditions so that the short primers can anneal with the DNA more frequently. These short primers, typically 10 nucleotides in length, produce multiple amplification products, visualised after electrophoresis as a series of bands. The band patterns vary according to the template. Perfect and/or imperfect inverted repeats which permit multiple mismatches and amplification are responsible for the banding pattern, and hence detecting the variation in DNA sequences at the priming sites of different genomes.

RAPD has been used as a tool for a variety of applications such as gene mapping (Honeycutt, et al., 1993), species identification and population analysis (Russel et al., 1993). It has been used extensively to identify species of bacteria (Akopyanz et al., 1992), fungi (Kersulyte, 1992), parasites (Bandi et al., 1993), insects (Dinesh, 1993), fish (Partis & Wells, 1996), plants (Williams, 1990), and mammals (Gwakisa, 1994). However, despite its extensive use in taxonomic identification (Akopyanz, et al., 1992) and phylogenetic studies, it has been used relatively rarely in human DNA research (Sineo, 1993).

A key factor in the use of RAPD is that no prior knowledge of the target is required, nanogram quantities of template can be used and results can be read directly from agarose gels based on the absence or presence of amplified DNA bands. Additionally, it can lead to direct sequencing of the amplified information without the use of fragment cloning or DNA strand separation. Finally and most significantly, it is far easier, safer and less expensive than corresponding techniques, such as Arbitrarily Primed PCR (AP PCR) and short tandem repeats (STR) detection.

Towyn-Y-Capel Human and Faunal Population Analysis

Samples of bones and teeth that were not clearly associated with specific skeletons (non-burial associated samples) were utilised for initial investigations into aDNA recovery from the Towyn-Y-Capel remains. The objective of this research was to assess the general condition of the material and the likelihood of successful aDNA retrieval from the remains of the greater collection.

Due to the lack of detail or structure on the random archaeological finds (non specific bone material from the site) which would allow the determination of the specific bone or species, RAPD (Randomly Amplified Polymorphic DNA) was used to gather some useful information on the random samples, as it does not require specific primer design. The results were initially quite poor (again teeth gave the best results), despite positive results with both controls and reference animal material. No improvements were achieved when a decalcification step was added to the Chelex protocol. However, results improved significantly upon extraction of DNA with the Qiagen mini blood kit and the Bio101 aDNA Kit. The best results were obtained with the latter. A complete RAPD profile of 6 primers was consequently produced using either BIO 101 aDNA kit extractions and the RAPD kit. Sample genetic profiles were compared against each other and against the standard profiles. Unfortunately no profile matches could be found (Results not shown).

Based on previous experience within the department of the identification of animal and human species with RAPD fingerprinting, initial analysis of samples was undertaken using RAPD as a simple and effective means of assessing the condition of genetic material. RAPD was a logical choice of technique, particularly as the lack of detailed bone/tooth morphology made species identification difficult. RAPD was selected as the tool of choice to gather genetic information from the random samples, as it can amplify DNA from unknown genomic material. DNA extracted from these materials was initially difficult to amplify, despite highly successful results from controls and reference animal material. These results were obtained using DNA recovered by Chelex extraction.

DNA amplification of the samples was not improved by the inclusion of a prior decalcification step. However, amplification of DNA was most improved by extraction of DNA with the QIAmp Mini Blood Kit (Qiagen) and with the Geneclean aDNA Kit, which produced the most pronounced and complex banding patterns. A complete RAPD profile of 20 remains using 6 different RAPD primers was consequently produced using the Geneclean aDNA kit and the RAPD kit (Figures 4.17-4.22). Sample genetic profiles were compared against each other and against the standard profiles (A selection of sample profiles produced by RAPD primer 4 are given in figures 4.23-4.25.

Unfortunately, no complete marker profile matches were found amongst the non-skeletal population studied or with the control species. Similar results were obtained for human DNA where variation in the template concentrations gave different banding patterns in all 6 primers (Figures 4.26-4.28). Correlation between human samples was equally sporadic for a selection of skeletal samples (Figures 4.29-4.32) using RAPD Primer 3.

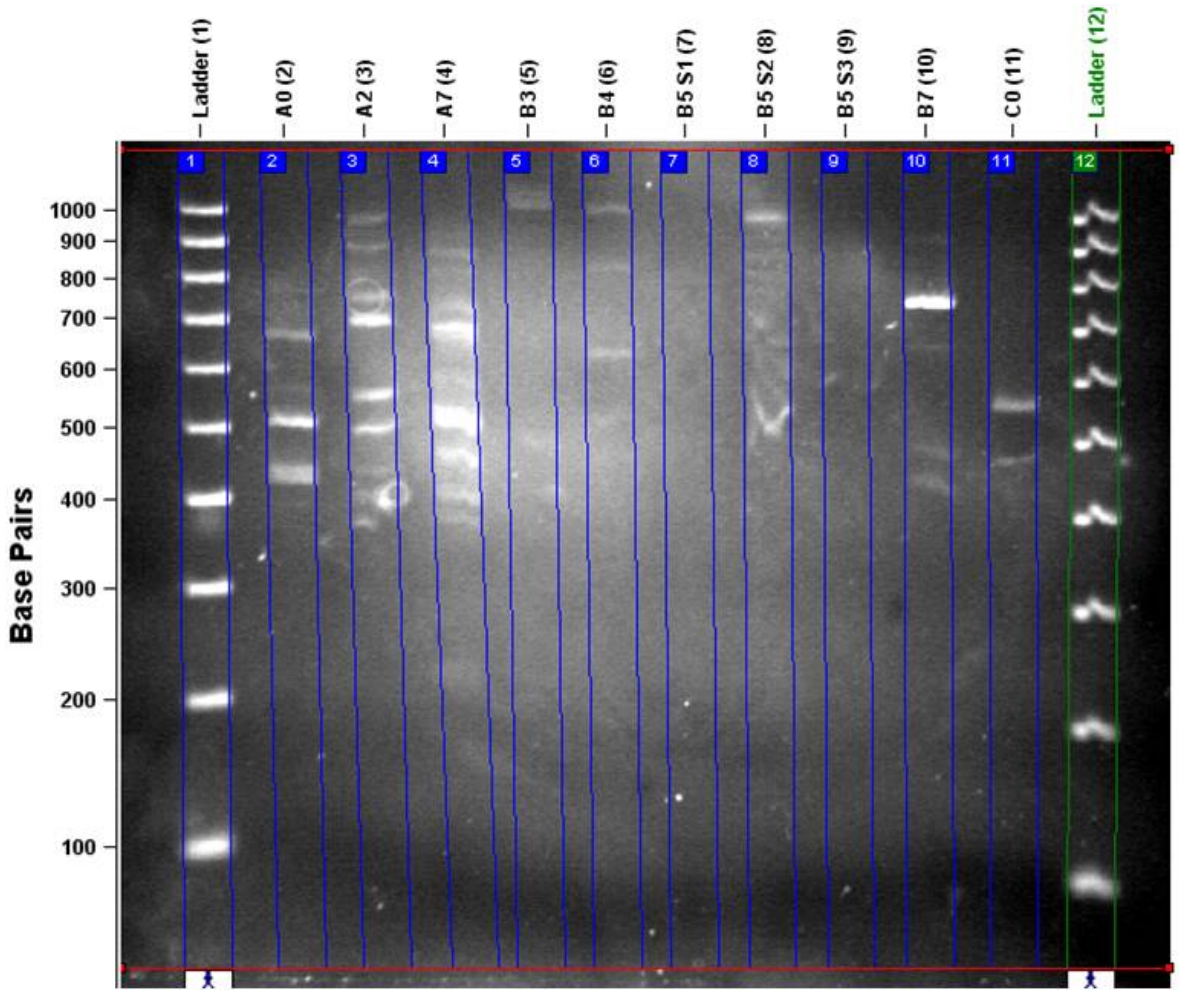


Figure 4.17: RAPD Profile of Random Finds using Primer 1.
Lanes 2-11 contained the samples A0, A2, A7, B3, B4, B5 S1, B5 S2, B5 S3, B7 & C0. Results typical of these electrophoresis runs.

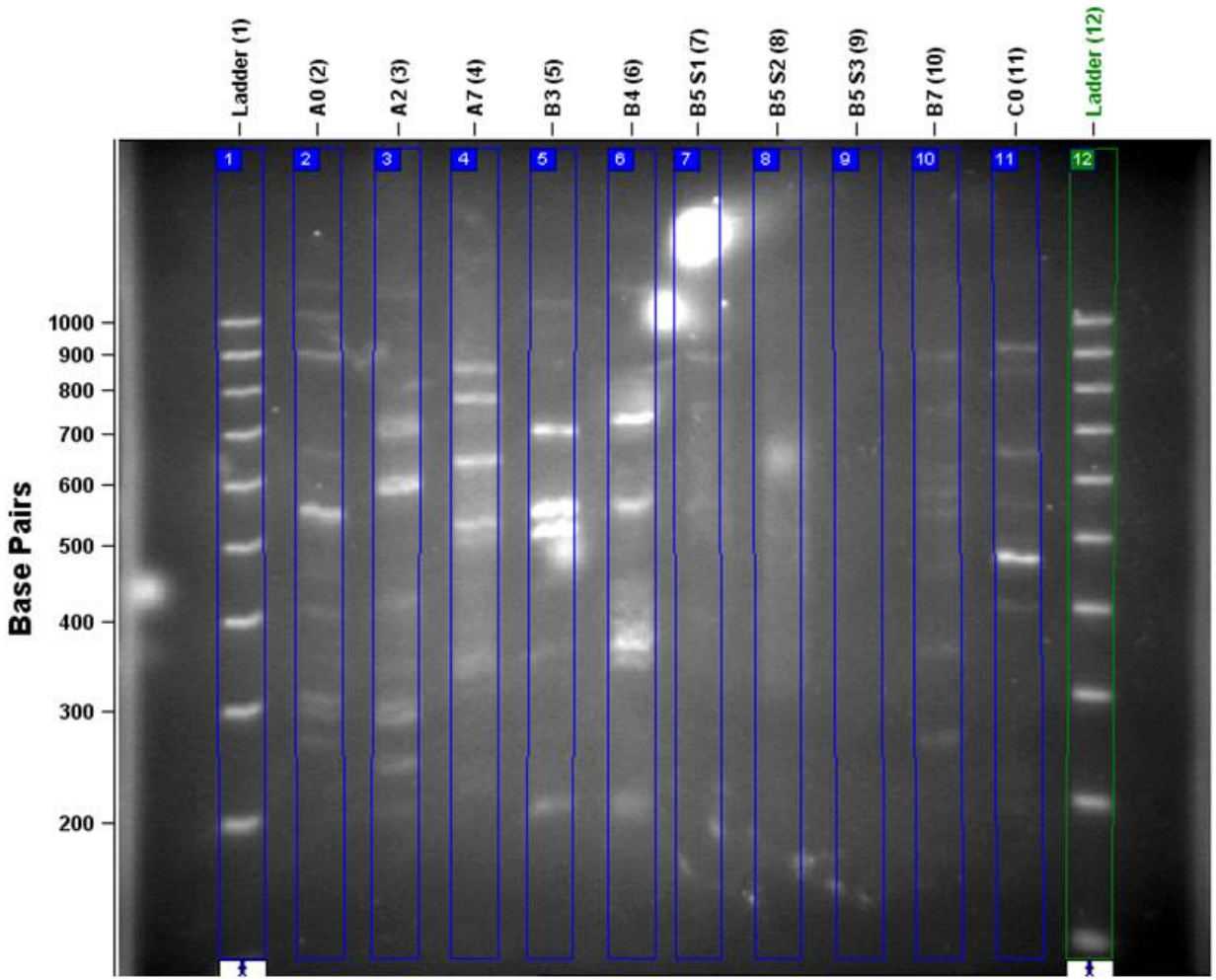


Figure 4.18: RAPD Profile of Random Finds using Primer 2.
Lanes 2-11 contained the samples A0, A2, A7, B3, B4, B5 S1, B5 S2, B5 S3, B7 & C0. Results typical of these electrophoresis runs.

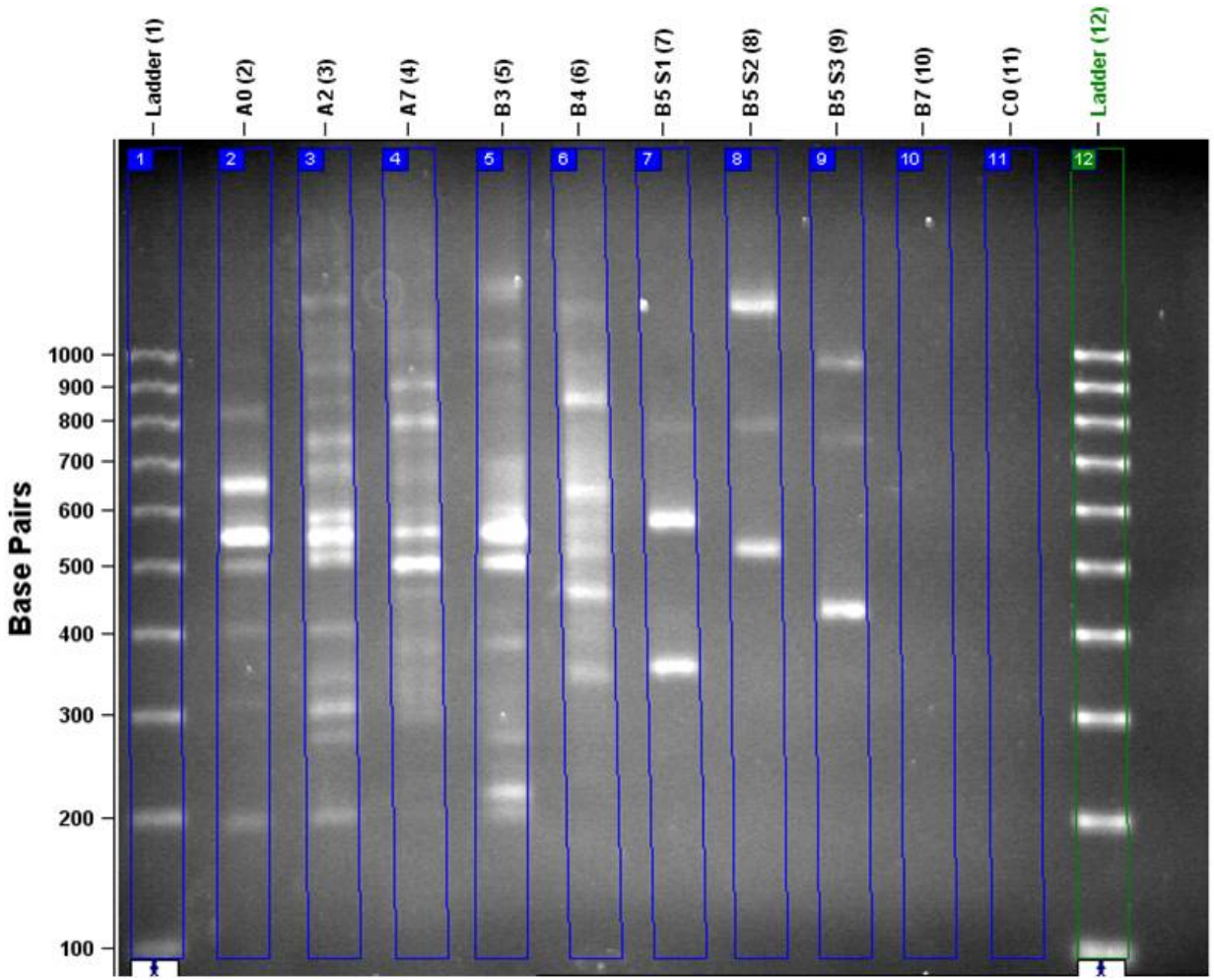


Figure 4.19: RAPD Profile of Random Finds using Primer 3.
Lanes 2-11 correspond to the samples A0, A2, A7, B3, B4, B5 S1, B5 S2, B5 S3, B7 & C0. Results typical of these electrophoresis runs.

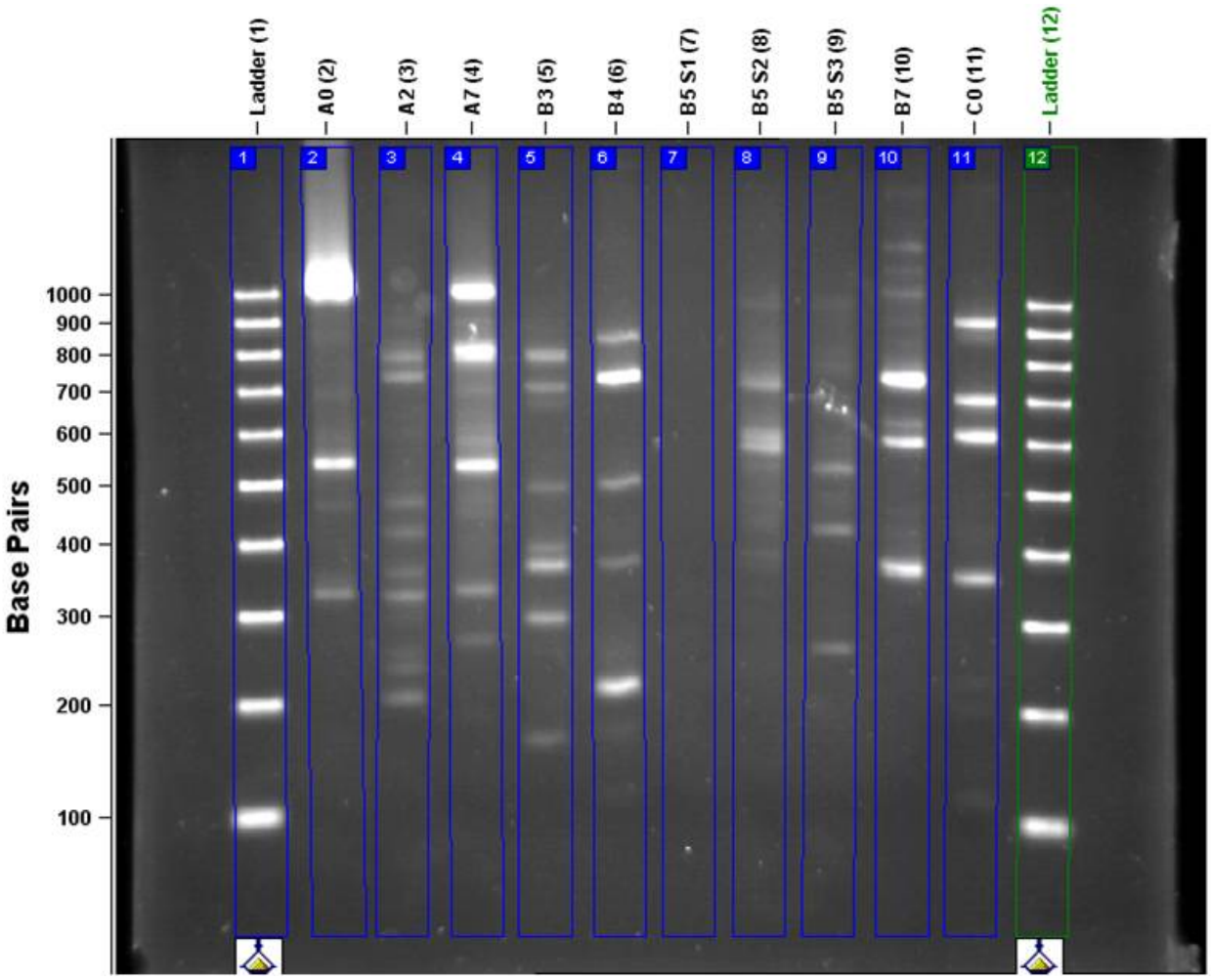


Figure 4.20: RAPD Profile of Random Finds using Primer 4.
Lanes 2-11 correspond to the samples A0, A2, A7, B3, B4, B5 S1, B5 S2, B5 S3, B7 & C0. Results typical of these electrophoresis runs.

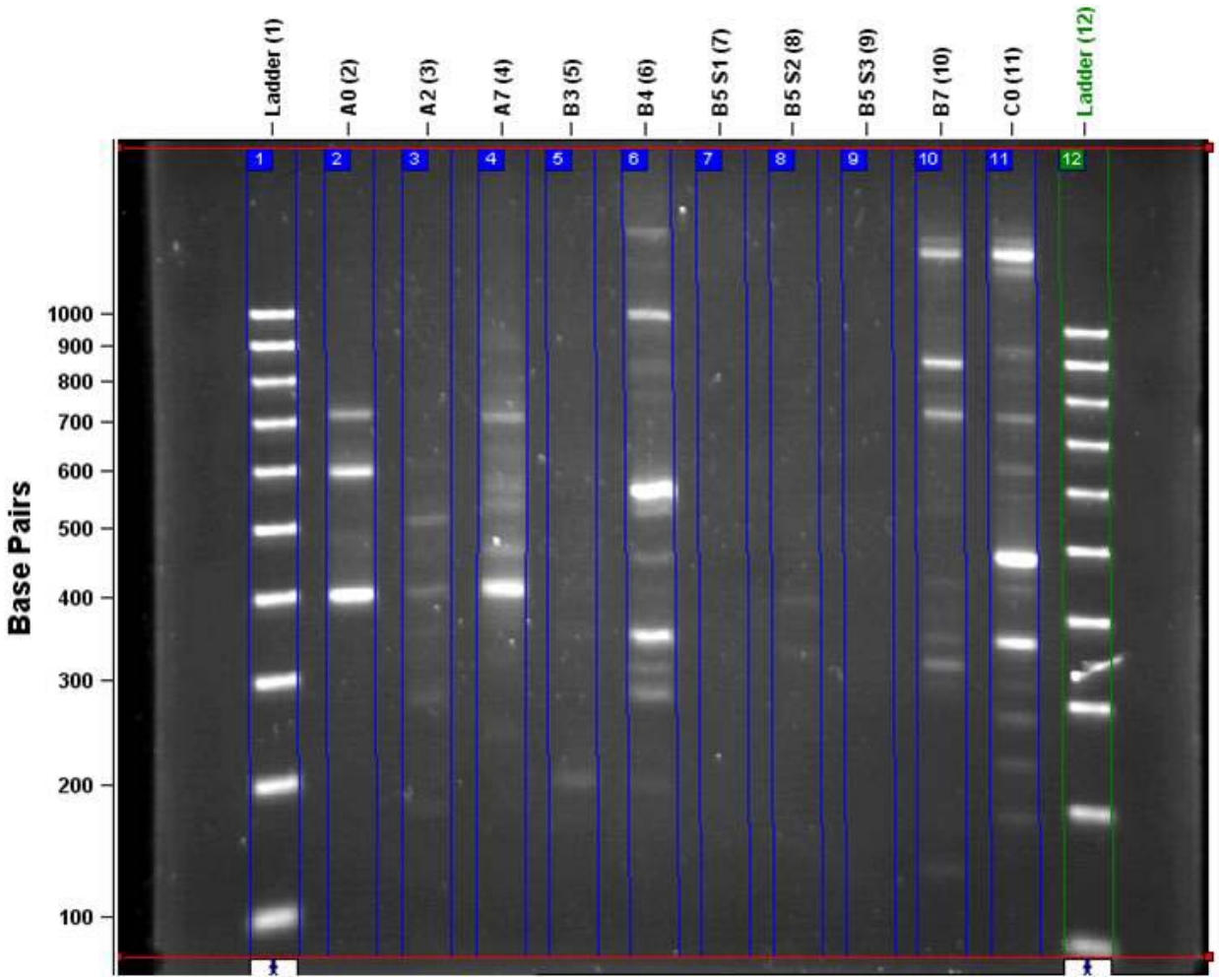


Figure 4.21: RAPD Profile of Random Finds using Primer 5.
Lanes 2-11 corresponds to the samples A0, A2, A7, B3, B4, B5 S1, B5 S2, B5 S3, B7 & C0. Results typical of these electrophoresis runs.

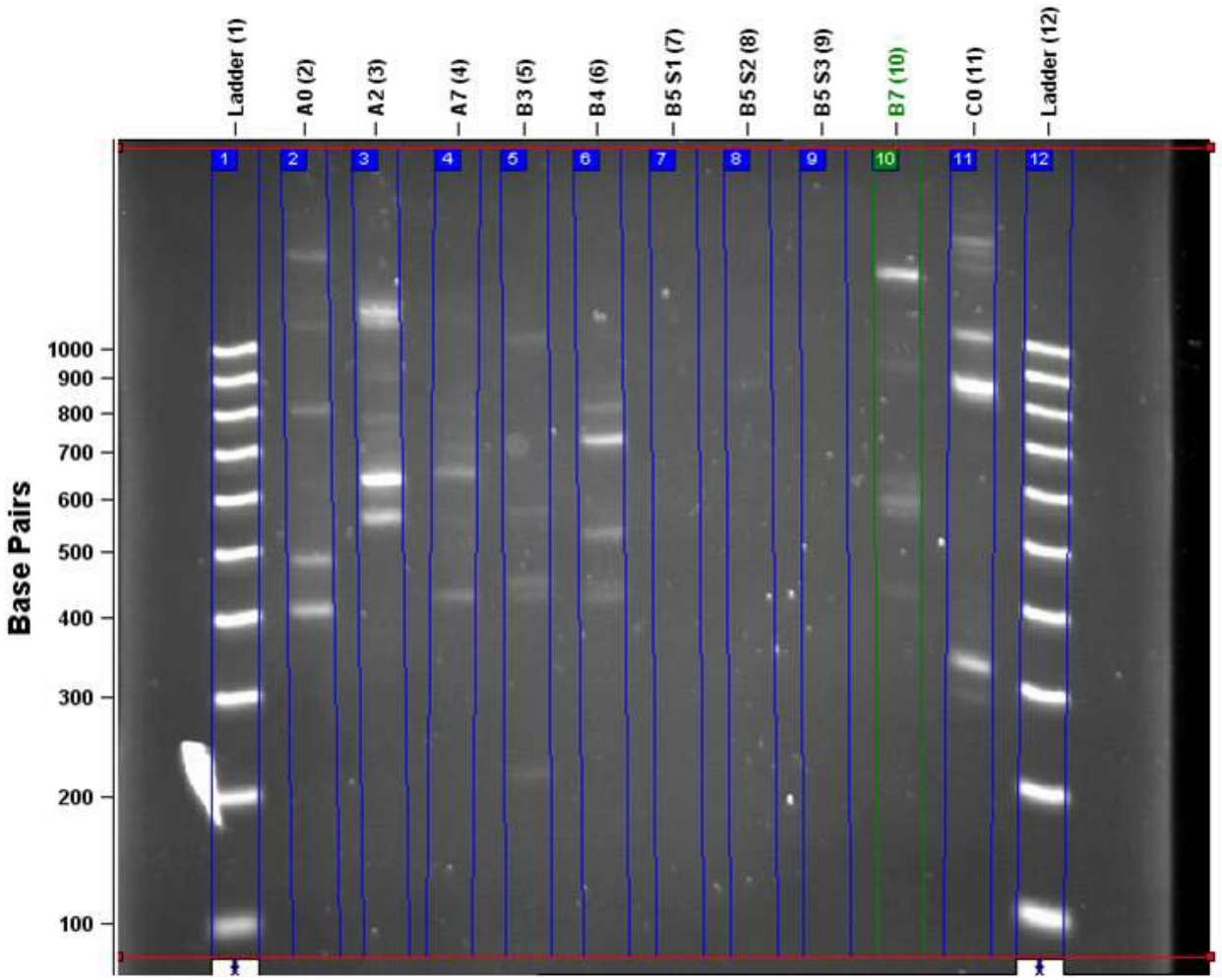


Figure 4.22: RAPD Profile of Random Finds using Primer 6.
Lanes 2-11 corresponds to the samples A0, A2, A7, B3, B4, B5 S1, B5 S2, B5 S3, B7 & C0. Results typical of these electrophoresis runs.

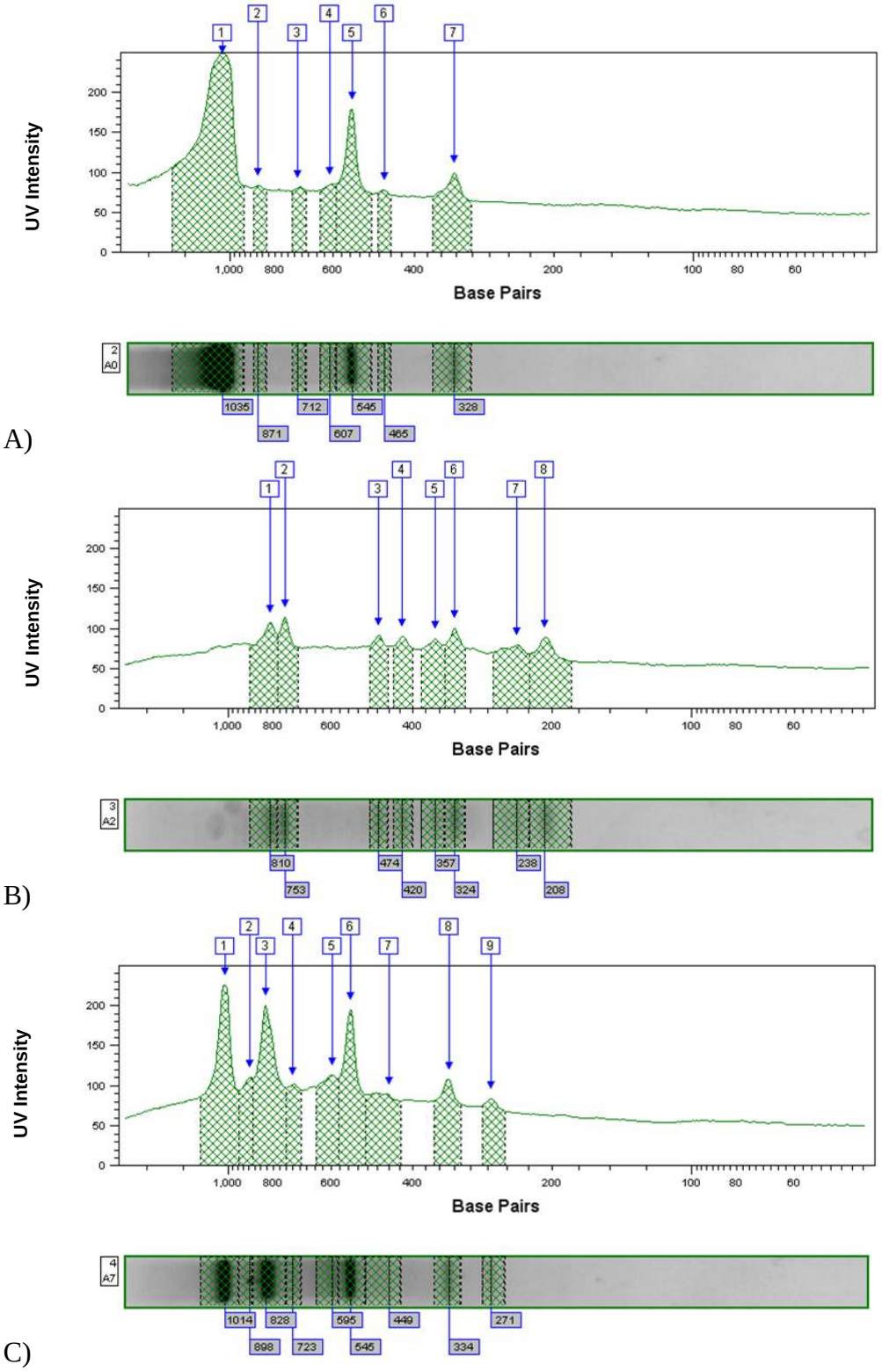


Figure 4.23: Individual Fingerprints for Each Sample with RAPD Primer 4 (Lanes 2-4).
Used for comparison of the banding pattern of samples A0 (A), A2 (B) & A7 (C). Peaks are measured according to two molecular size ladders, band size is estimated (in base pairs) and peaks measured as direct UV intensity (Arbitrary Units).

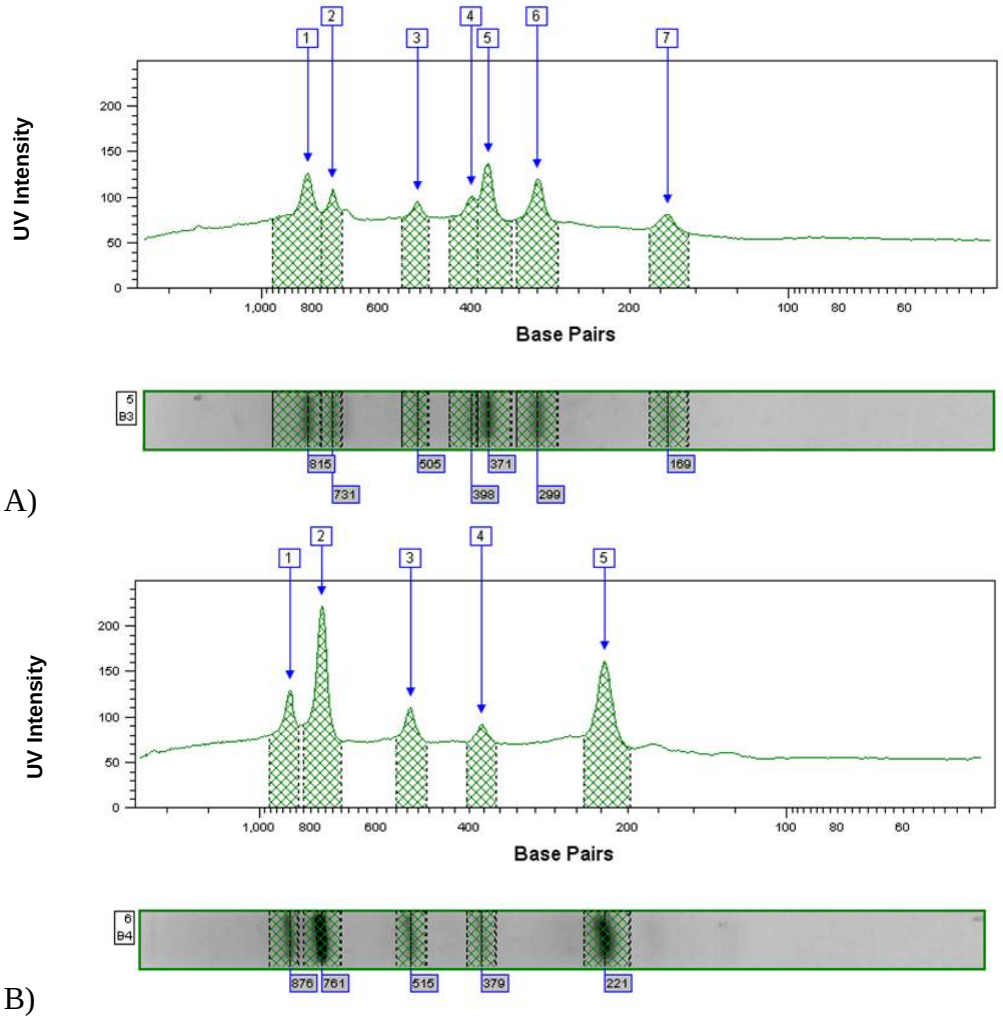


Figure 4.24: Individual Fingerprints for Each Sample using RAPD Primer 4 (Lanes 5-8).
Used for comparison of the banding pattern of samples B3, B4 (A) & B5 S3 (B). Peaks are measured according to two molecular size ladders, band size is estimated (in base pairs) and peaks measured as direct UV intensity (Arbitrary Units).

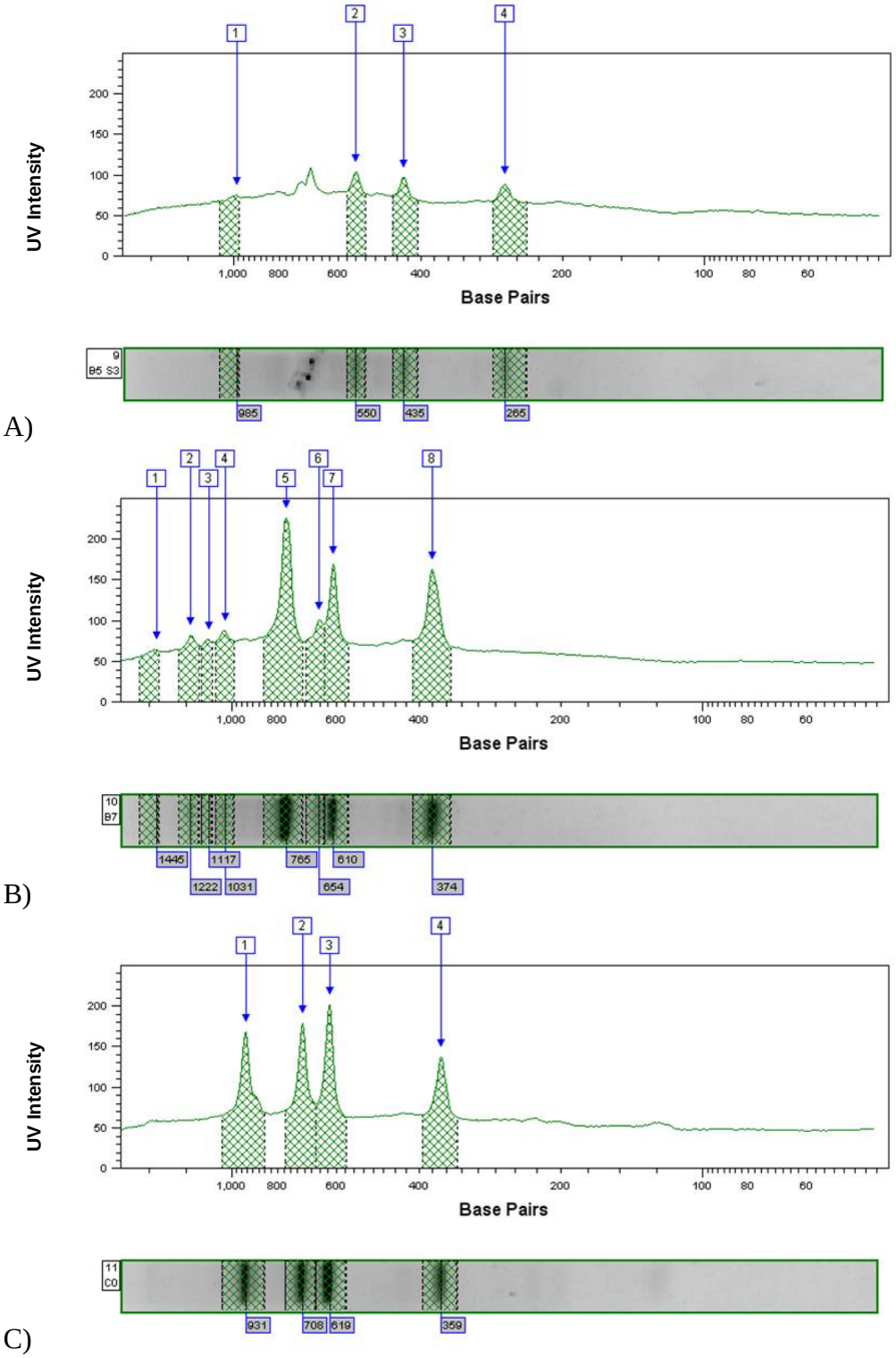


Figure 4.25: Individual Fingerprints for Each Sample using Primer 4 (Lanes 9-11).
Used for comparison of the banding pattern of samples B5 S3 (A), B7 (B) & C0 (C). Peaks are measured according to two molecular size ladders, band size is estimated (in base pairs) and peaks measured as direct UV intensity (Arbitrary Units).

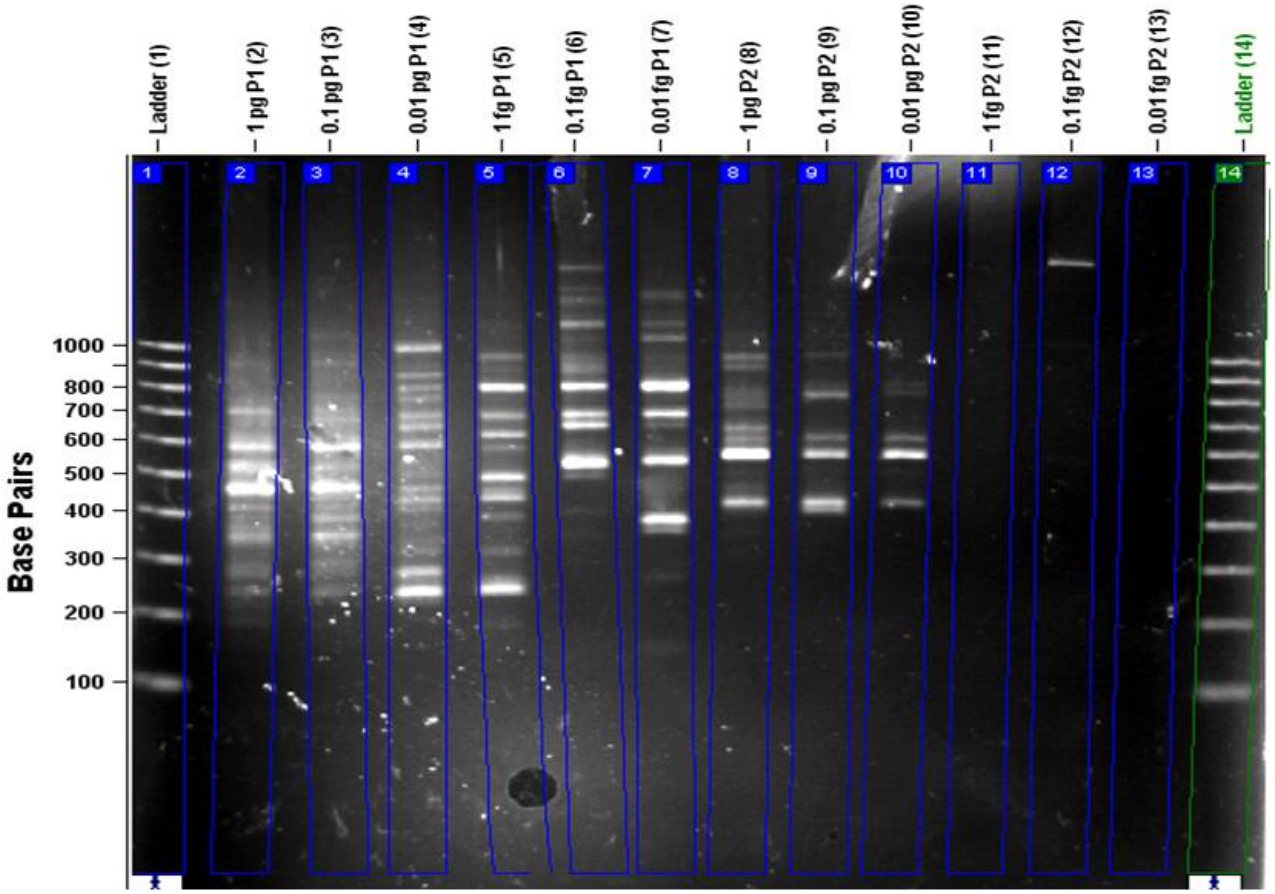


Figure 4.26: RAPD of Human DNA Dilutions using Primers 1 &2.
Dilutions of 1 ρg to 0.01 *f*g of modern fragmented human DNA. Results typical for these experiments.

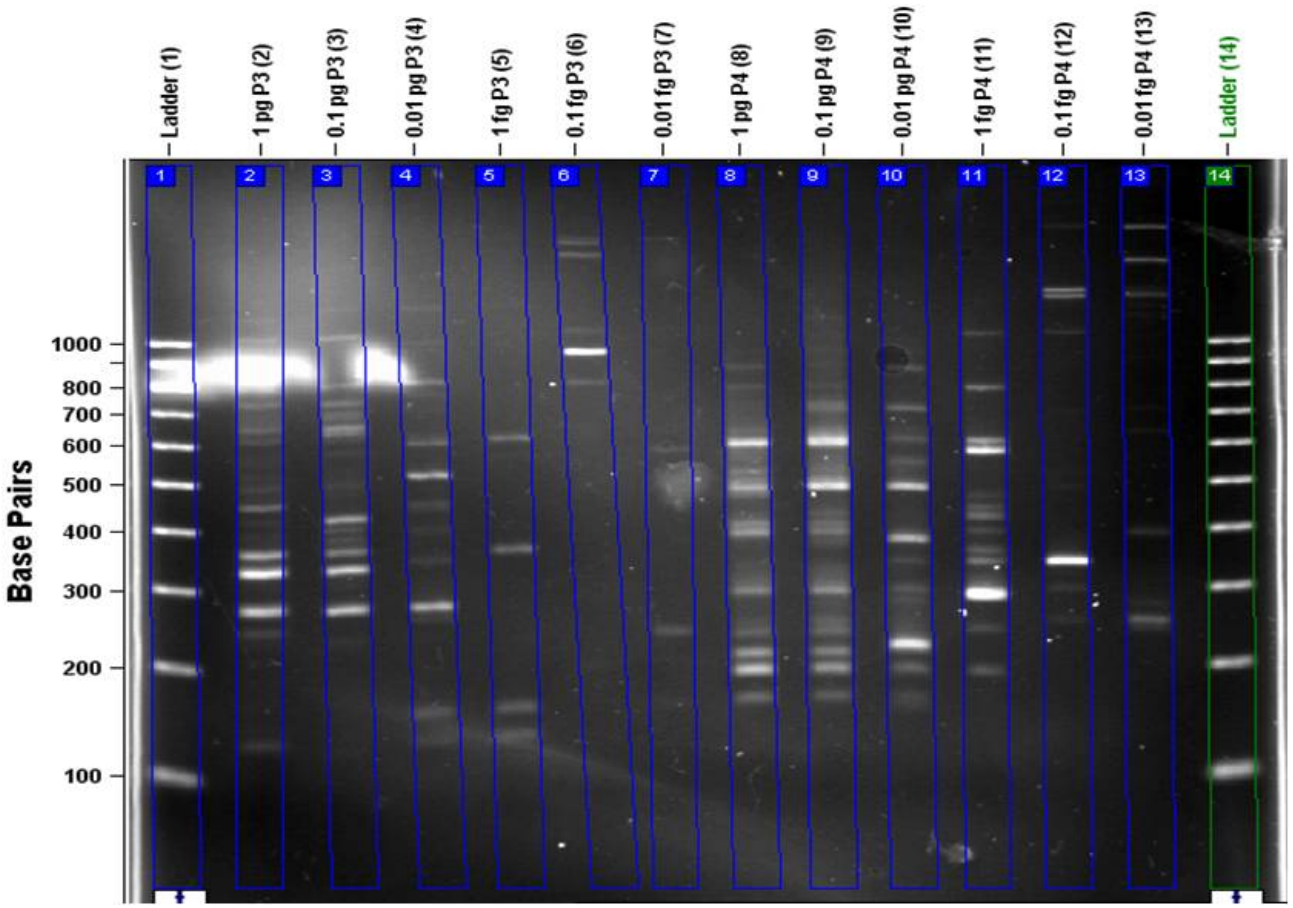


Figure 4.27: RAPD of Human DNA Dilutions using Primers 3 & 4.
Dilutions of 1 ρg to 0.01 *f*g of modern fragmented human DNA. Results typical for these experiments.

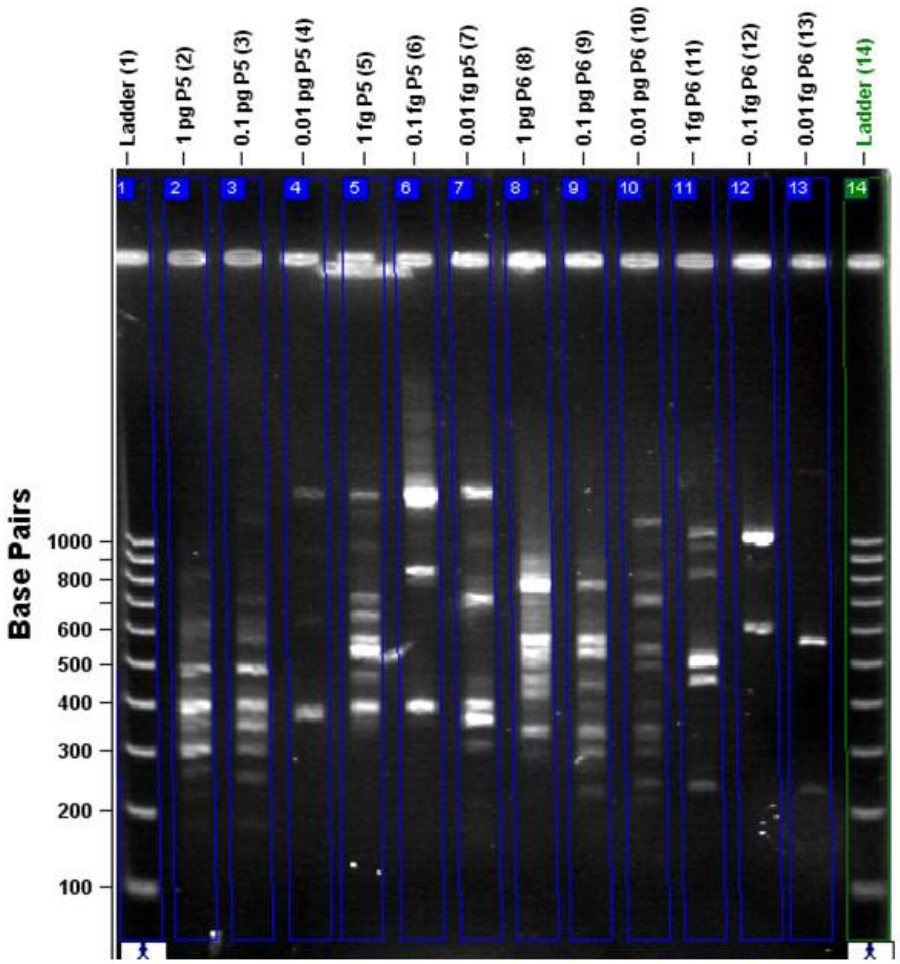


Figure 4.28: RAPD of Human Control DNA Dilutions with RAPD primers 5 & 6.
Dilutions of 1 ρg to 0.01 *f*g of modern fragmented human DNA.

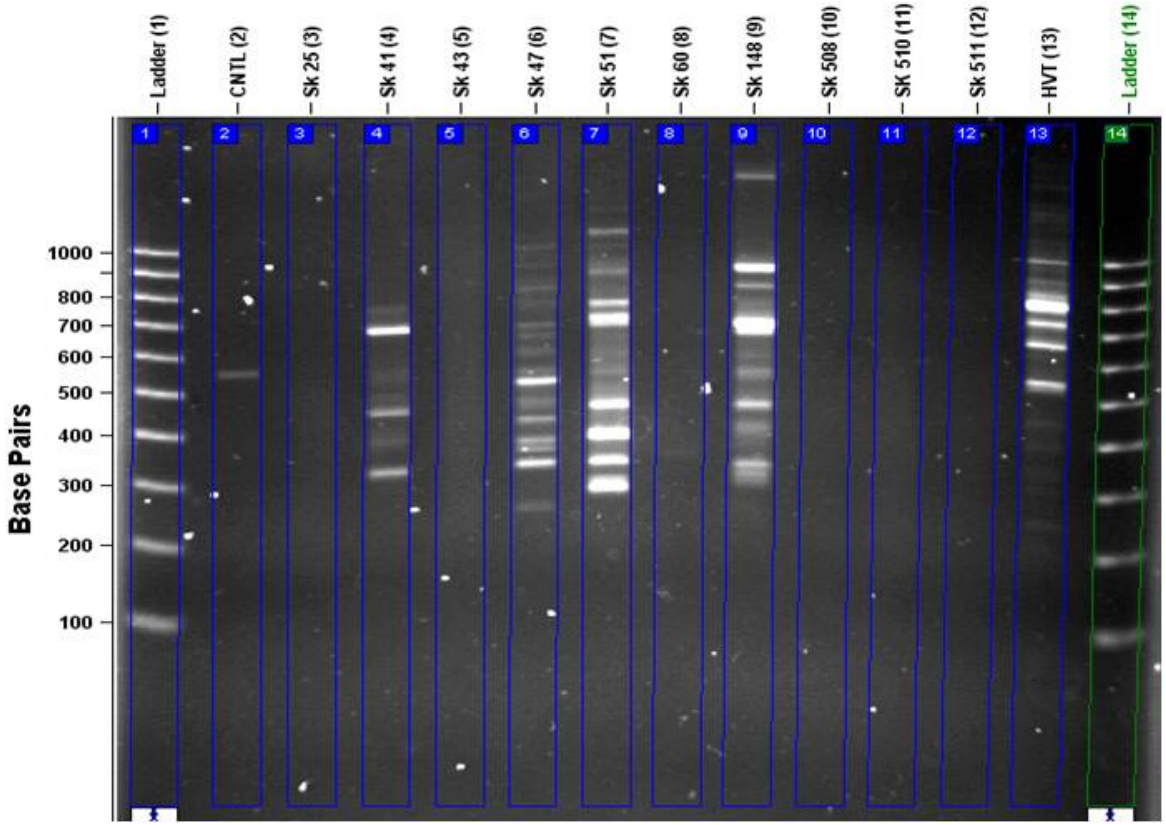


Figure 4.29: RAPD Profile of Human Skeletal Samples with Primer 3.
RAPD fingerprints of skeletal samples Sk25, Sk41, Sk43, Sk47, Sk51, Sk60, Sk184, Sk508, Sk510, Sk511 and from a control sample a human Victorian age tooth (HVT).

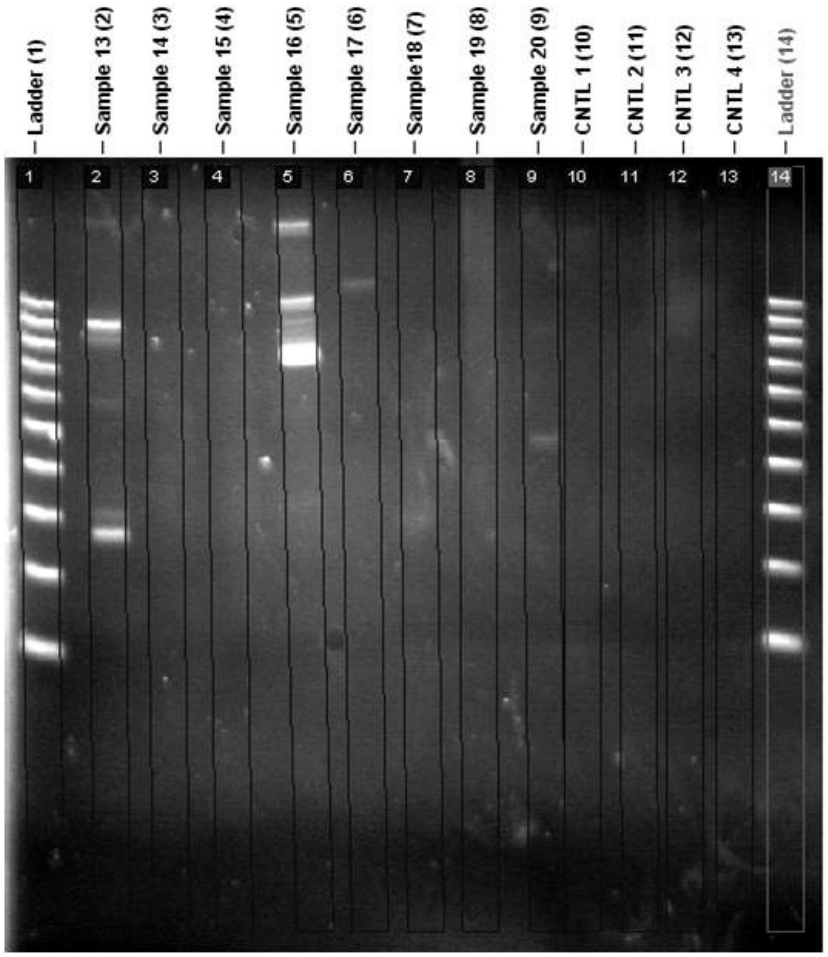


Figure 4.30: RAPD Profile of Human Skeletal Samples with Primer 3.
RAPD fingerprints of skeletal samples SK508a, Sk508b, Sk510, UkB, UkC, HA1, HA3 and a negative extraction control (samples 13-20 sequentially). CNTL 1-4 are the controls for human RAPD reactions (Figures 4.29 & 4.30).

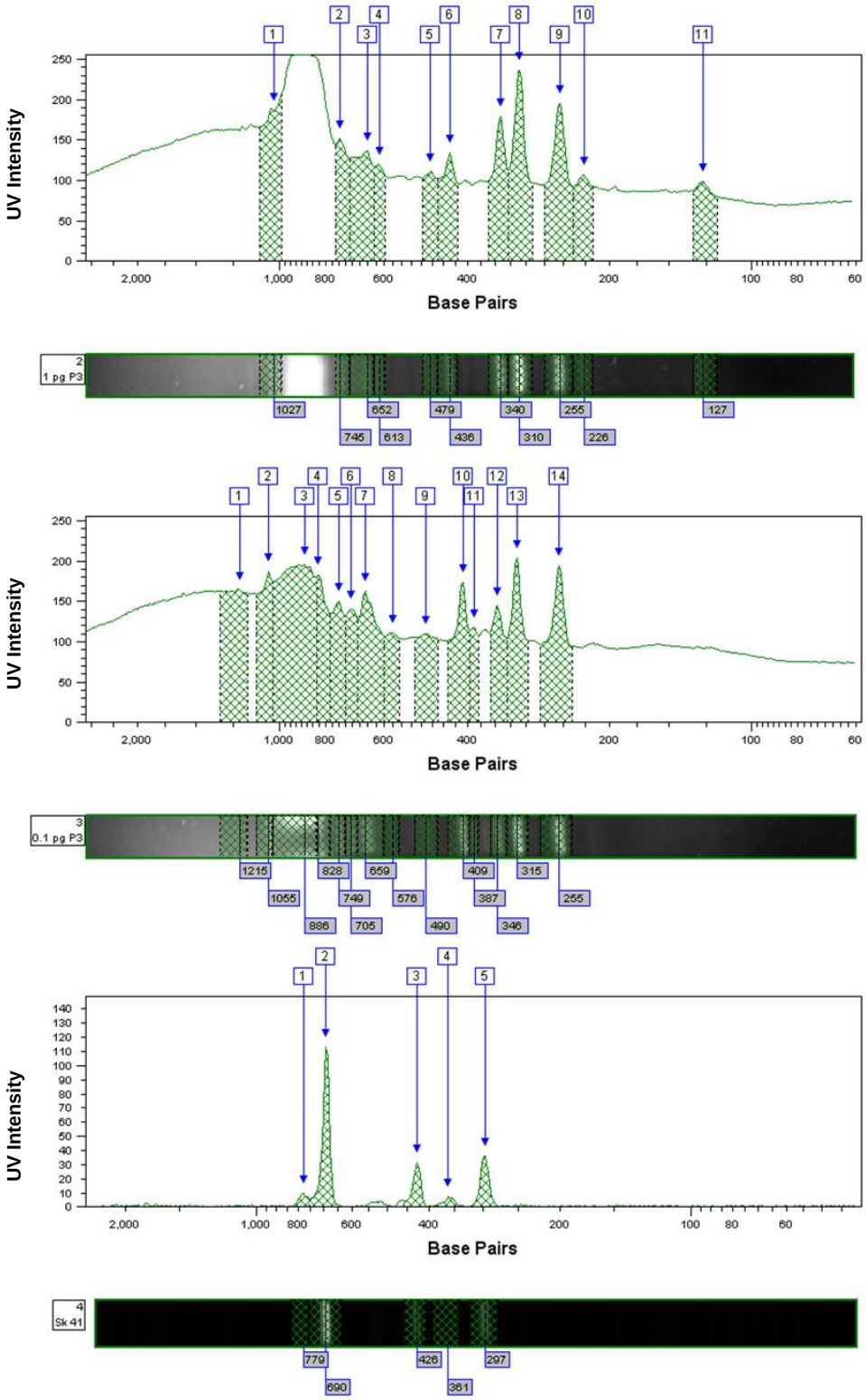


Figure 4.31: Individual Fingerprints for Human Controls (Dilutions) & Sample Sk41.
Individual fingerprints of each sample (lane) showing the molecular size of the bands and intensity for comparison. Samples corresponding to lanes 2, 3 and 4 are pictured. Lanes 2 and 3 are human DNA dilution fingerprints using RAPD primer 3, lane 4 corresponds to the fingerprint produced by Sk41 with the same primer.

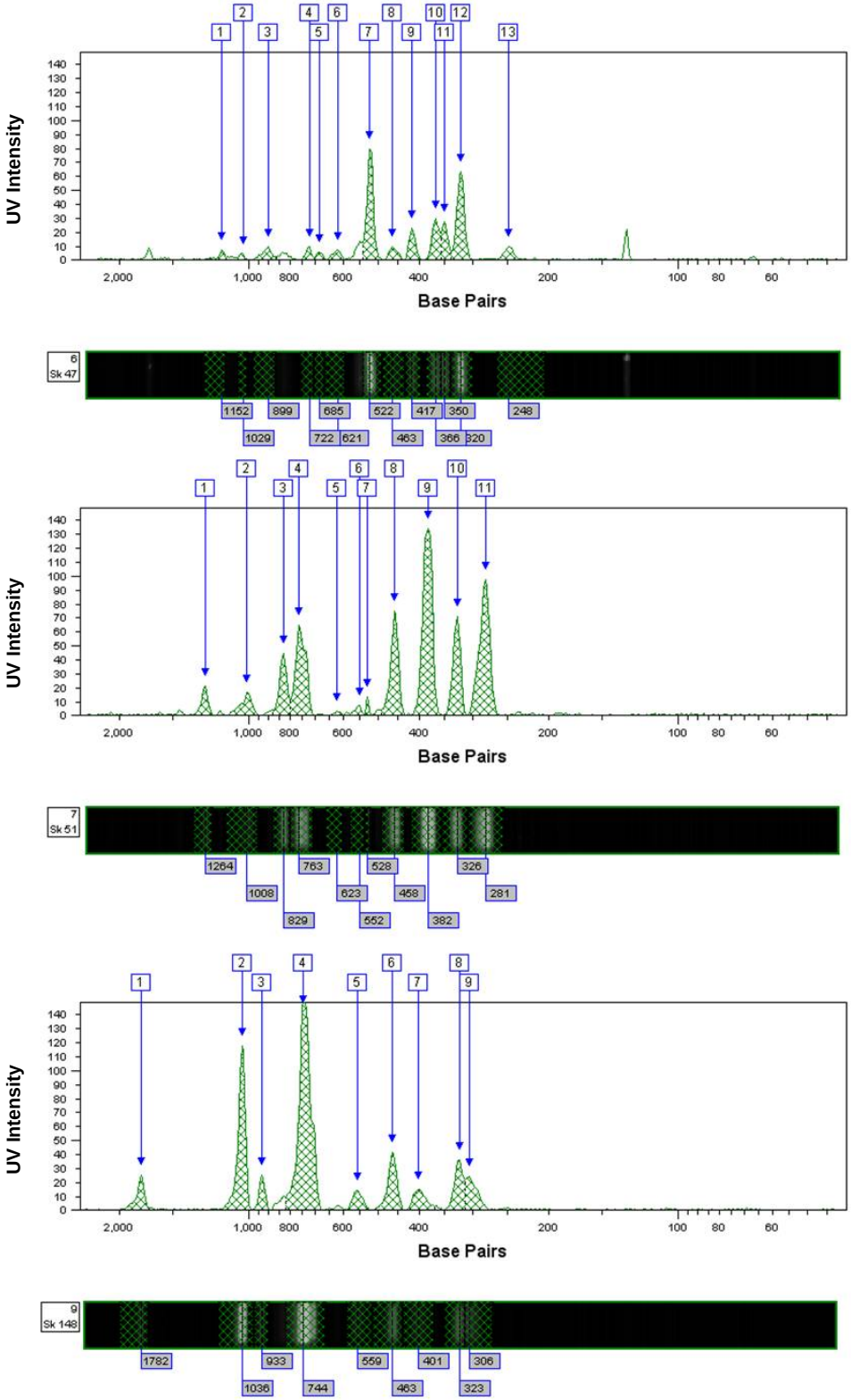


Figure 4.32: Individual Fingerprints for Samples Sk47, Sk51 & Sk148 with RAPD Primer 3.
Used for comparison of the banding pattern of each sample, peaks are measured according to two molecular size ladders, band size is estimated (in base pairs) and peaks measured as direct UV intensity (Arbitrary Units).

4.5.5 Mitochondrial DNA specific Primer Selection and Optimisation

Following on from the RAPD experiments, a more specific approach was attempted to amplify population-specific genetic material using human DNA specific primers. These were selected to amplify fragments of human mitochondrial DNA, and in particular the hypervariable region 1 (HV1) of the D-loop of the mitochondrial genome, and the hypervariable region 2 (HV2). These sites were chosen because of the high variability of the mitochondrial sequence in these areas and the large databases of sequences in these areas available. Specific primers were selected from the literature to meet certain conditions, primarily as overlapping primers allow the complete sequencing of a hypervariable region. These could also be run in unison (i.e. as a multiplex) in order to rapidly screen potential targets and assess the viability of certain primers within the multiplex.

Preliminary screen and contamination control

A preliminary assessment was carried out using loose find (non-skeletal material) samples found at the first burial site exhumed. These samples were putatively identified and classified by Michael Wykoski and Rosa Spencer for bone type and species. These samples were unable to be identified as human, and were therefore probably a mixture of human and animal skeletal fragments. The objective of this preliminary assessment was to check the robustness of collection and sampling procedures for avoidance of contamination of ancient samples by modern human DNA and cross-contamination of individual ancient samples from endogenous sources, such as plant material or other bone or tooth samples at the site.

Using these data, a selection of samples was chosen to verify the robustness of laboratory procedures for the extraction and amplification of human DNA using human mitochondria specific primers for a 424 bp product. Moreover an initial screening test was employed before the continuing excavations were conducted at the burial site in 2002/2003. Initial screening of random DNA samples was undertaken using samples obtained with Chelex DNA extraction, but this was later substituted with Bio 101 and Qiagen extraction methods.

The Results were completely negative for all bone samples and negative controls but positive results were obtained with human DNA controls and for some tooth samples of human origin from the site.

Unfortunately, the latter proved to be not consistently reproducible Samples of limited archaeological/anthropological importance (animal or non-descript bone fragments) taken from the archaeological site were used for optimisation and assessment assays of extraction and amplification, and were preliminarily screened for modern contamination using the L29 and H408 primer set. These produce large fragment sizes, and are hence unlikely to amplify ancient DNA due to the fragmented nature of the aDNA template (usually resulting in products not larger than 200-300 bp) (see Figures 4.33-4.34). As part of the selection process, estimates of the primer details, in particular the optimal annealing temperature, were calculated using oligonucleotides properties calculator[4].

Mitochondrial DNA HV1 Specific Gerstenberger Primer Set

This multiplex set of six primers was designed to amplify four specific sites within the hypervariable D-loop or control region of the human mitochondrial genome. The advantage in choosing this primers set is that the primers overlap, which allows sequencing of the entire HV1 area which, as previously stated is the main hypervariable region used in determining mitochondrial haplotypes. In addition, a part of the HV2 region, whilst remaining under the theoretical 250 bp aDNA size limit (131 bp, 168 bp, 217 bp) is used as a single multiplex able to amplify all these regions simultaneously, with advantages as a screening reaction. In addition to this, the co-ordination of two of the primer sets allows for a larger 313 bp product to be amplified, which is an indicator of the gross condition of the DNA template (shown as a fourth, larger band, on the gel (Figure 4.35).

This amplification reaction was optimised for different annealing temperatures, $MgCl_2$ concentrations, cycle profiles, primer concentrations and buffers (including Parr, formamide, BSA, where not already present in the reaction mixture, and betaine, using 1 ρg amounts of modern human DNA. Furthermore, externally confirmed human DNA (Batch controlled 1 µg sonicated human DNA, Mobio) concentrations were used to verify the detection limit of this assay for human mtDNA. Figure 4.35 demonstrates these results with the assay optimised at the set profile and mixture sensitivity in this assay was between 10 and 100 fg. However, at these low concentrations the smallest base-pair band does not appear to amplify Due to the large size of the fragment, as expected in aDNA.

[4] www.basic.nwu.edu/biotools/oligocalc.html

To evaluate the extraction technique and the presence of contamination, 12 non-skeletal samples were assayed for the presence of human mitochondrial targets (Figures 4.33-4.34; 4.36-38). Of these samples, three were suspected to be of human origin. The results confirmed human origins in two cases and one sample that did not appear to be human was amplified and one sample that appeared to be human did not. All other samples yielded negative results. The amplification of these products showed the presence of the lower two and in one case the lower three, bands of the multiplex, indicating a degraded template DNA.

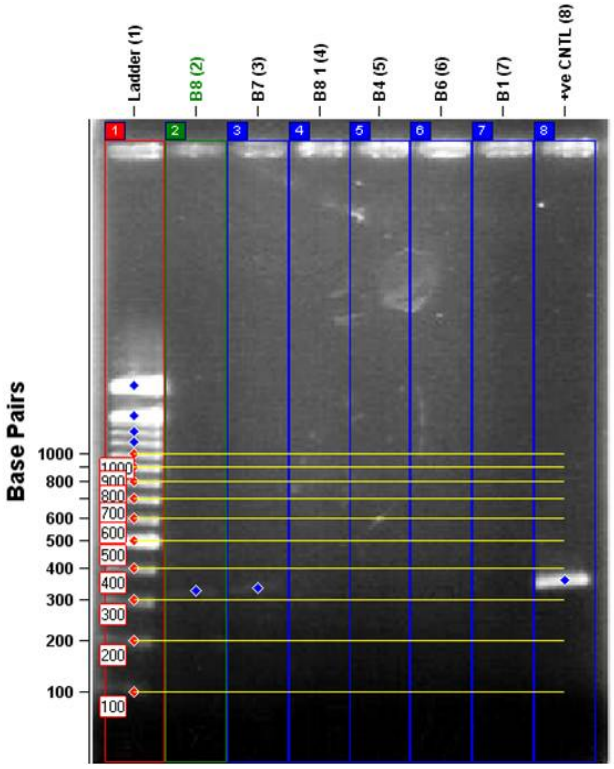


Figure 4.33: (Left) **mtDNA Contamination Trials.** mtDNA specific primers L29 and H408 amplify a 412 bp product.

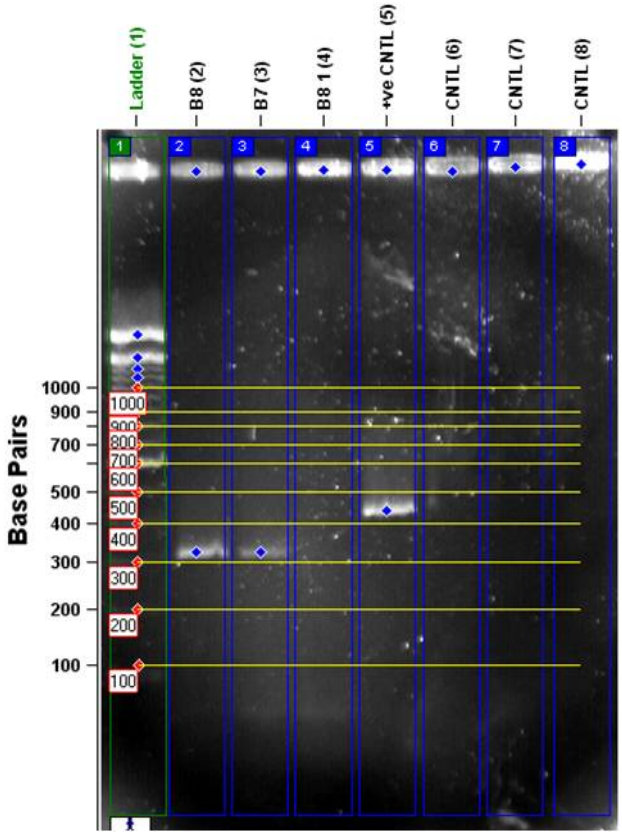


Figure 4.34: Contamination Assessment of Towyn-Y-Capel samples.
Preliminary assessment of DNA preservation in Towyn-Y-Capel samples. Primers used were the same L29 and H408 primers specific for a 412 bp product, used for contamination assessment. Samples 1-6, in Lanes 2-7; Lane 1, 100 bp ladder; Lane 8, positive control.

4.5.6 Mitochondrial DNA Specific Amplification of the HV1 region in the Population samples from Towyn-Y-Capel using the Gerstenberger Primer Set

The Gerstenberger multiplex human mtDNA reaction was used in conjunction with the optimised extraction technique (phase 1 extraction conditions) to initially screen random samples for human DNA. This was accomplished with some success, with 3 successful amplifications from 15 samples analysed. Two of these corresponded to human samples, while one did not, and two other human samples appeared to give negative results. The Gerstenberger multiplex reaction should yield 4 amplicons, with the largest amplicon being a good indicator of the condition of ancient DNA. The large amplicon (312 bp) is more likely to amplify when the sample DNA is in very good condition or as a result from modern contamination. In this study, the positive results yielded only the lower three bands of 217, 168 and 131 bp.

Spiked samples were then set up by including modern human DNA in an extract from ancient skeletal samples. The results from these samples showed no amplification. This indicated that there was inhibition of the PCR in these samples, presumably as a result of humic or other inhibitory materials in the extracted solution. Consequently, an improved PCR master mix (Amplitaq™) was selected which, in the trial stages, had proved to be far superior to the standard PCR master mix (Reddytaq™).

Next, 20 human skeletal samples (Table 4.4) were assayed for the presence of the four human specific mitochondrial targets using the Gerstenberger primers. The results showed that, of the 20 known human samples, only one was amplified (HA 7) (Figures 4.36), which yielded only the smallest of the four possible amplicons. This result was verified by a second round of amplifications of the same samples, which also showed a single amplicon of the same size for sample HA7.

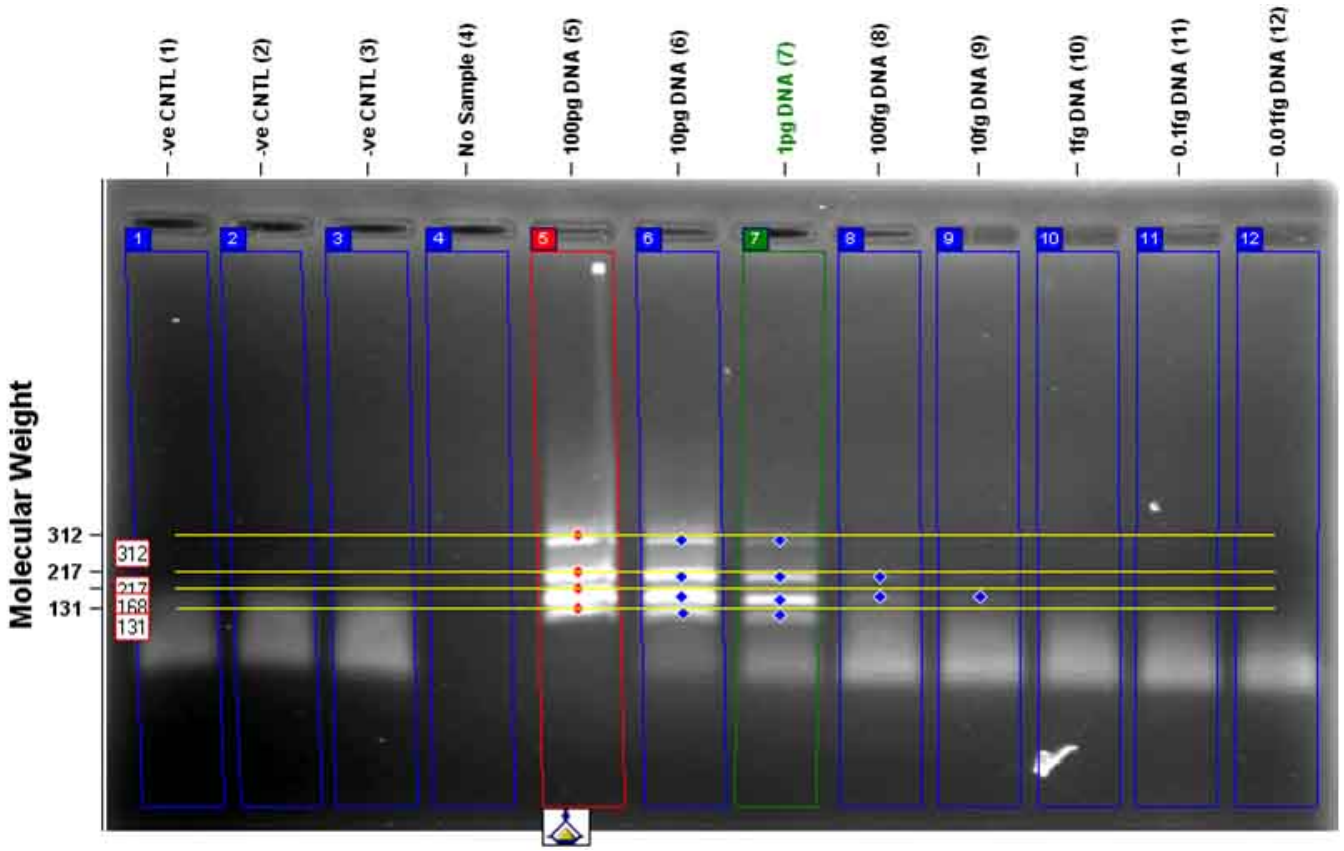


Figure 4.35: Gerstenberger mtDNA Primer Multiplex Limit of Detection Assay.
DNA dilutions were made of a picogreen dye quantified modern DNA standard. DNA was amplified using the mtDNA specific multiplex primers specific for four different products of varying sizes. Gel shows the electrophoretic gel of the range of DNA dilutions. Overlaid is the electrophoretic gel analysis, lane description and molecular weights of the multiplex bands.

To verify multiplex results and sensitivity, all 21 samples were analysed using only the two primers specific for the smallest target of the multiplex (primers H16233 & L16317 {Table 4.7}). The results were overwhelmingly different (Figures 4.39 & 4.43). This time, seven samples (HA 4, Sk17, HA7, HA8, Sk511a, Sk511 b and Sk516) all yielded positive results for the smallest amplicon. These results show that the efficiency of amplification was greater with the singleplex reaction, rather than the multiplex.

Upon repeating this amplification series and further analysis, however, there was a significant lack of results obtained for both the positive samples achieved before and the remainder of phase 1 extraction samples. Using both a wider range of template dilutions and increasing the number of PCR cycles from 35 to 45 and two different buffers (Amplitaq Gold® and GeneAmp® Gold Buffer mix), none of the extracts from phases 1-5 were amplified. Positive results were obtained with both the control extractions. The decision was therefore taken to use individual primer sets in singleplex reactions, to avoid competition between the primers singleplex amplification reactions using Gerstenberger primers H16233 & L16317 set on ancient population samples.

The greater sensitivity of the singleplex, compared to the multiplex, reaction was verified using 20 human skeletal samples in two independent singleplex reactions (Figures 4.42 & 4.43), which yielded amplification of seven positive samples for a 131 bp mtDNA fragment, namely Skeletons Sk25, Sk33, Sk41, Sk43, Sk51, Sk 60 and Sk 184. Figure 4.43 confirms the increased sensitivity of this reaction compared to the multiplex reaction, where only 3 of the 4 samples detected in the singleplex yielded amplicons. Figures 4.39 and 4.40 show the single lane comparison of the multiplex banding pattern and intensity compared to the 1 ρg modern DNA control.

Unfortunately, sequential singleplex amplification reactions for each of the primer sets (H16233/L16317) performed on extracts from phase 3-5 failed to produce successful results, despite positive and negative controls functioning correctly. In some reactions, a primer dimer was observed. Phase 1 and 2 tooth extracts were re-amplified with the smallest primer set (131 bp H16233/L16317), but without success. The poor performance of the amplification technique, despite optimisation, led to two conclusions: either the extracts did not have sufficient intact DNA molecules for mtDNA amplification and aDNA templates are being outcompeted by exogenous DNA, or that the PCR itself was stopped by the presence of PCR inhibitors. Since the extracts from phase 1, 2 and from random bone finds had already established the existence of DNA in these samples, the second hypothesis was considered most likely.

The success of the RAPD amplification of samples is at odds with the idea that lack of success was due to inhibition of the PCR, unless the inhibition was related to the endogenous DNA itself. This might arise from the presence of excised bases and Maillard reaction products. To counter this possibility PTB was added to all decalcification solutions for aDNA extracts from phase 3 to 5.

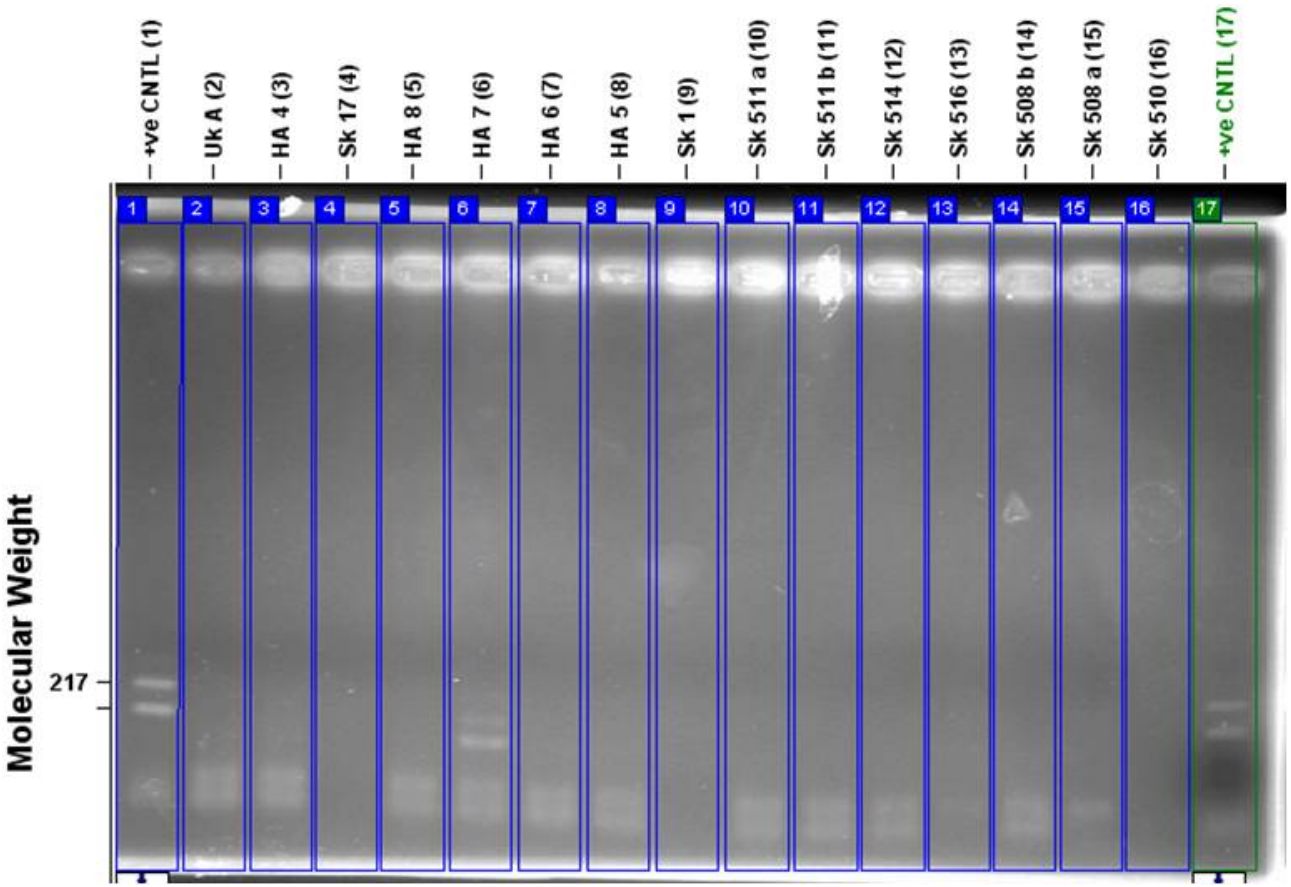


Figure 4.36: Gerstenberger mtDNA Multiplex Detection Assay for Human Samples 1-15; UkA, HA4, Sk17, HA8, HA7, HA6, HA5, Sk1, Sk511a, Sk511b, Sk514, Sk516, Sk508a, Sk508b & Sk510.

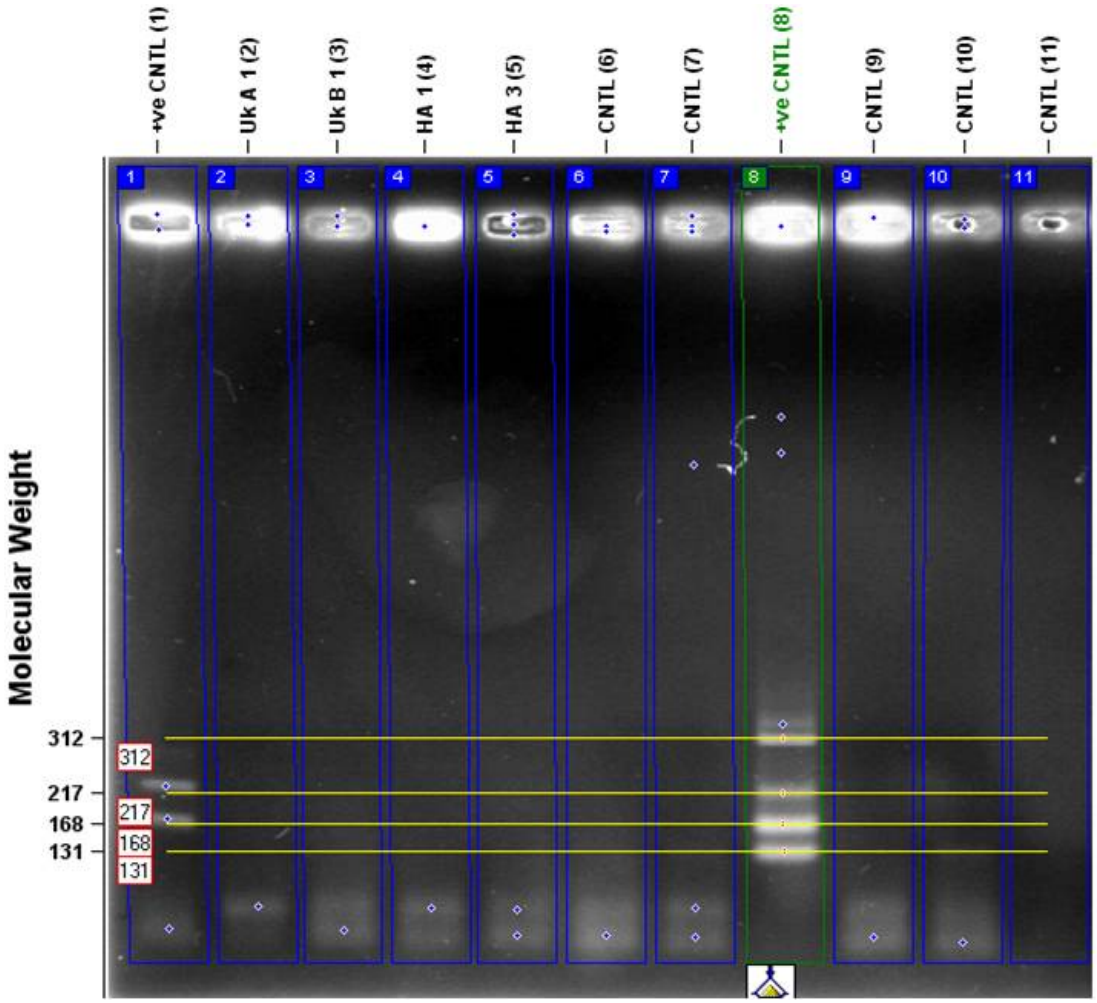


Figure 4.37: Gerstenberger mtDNA Multiplex for Human Samples 16-21; UkA1, UkB1, HA1, HA3 & Controls for Figures 4.36-4.37.

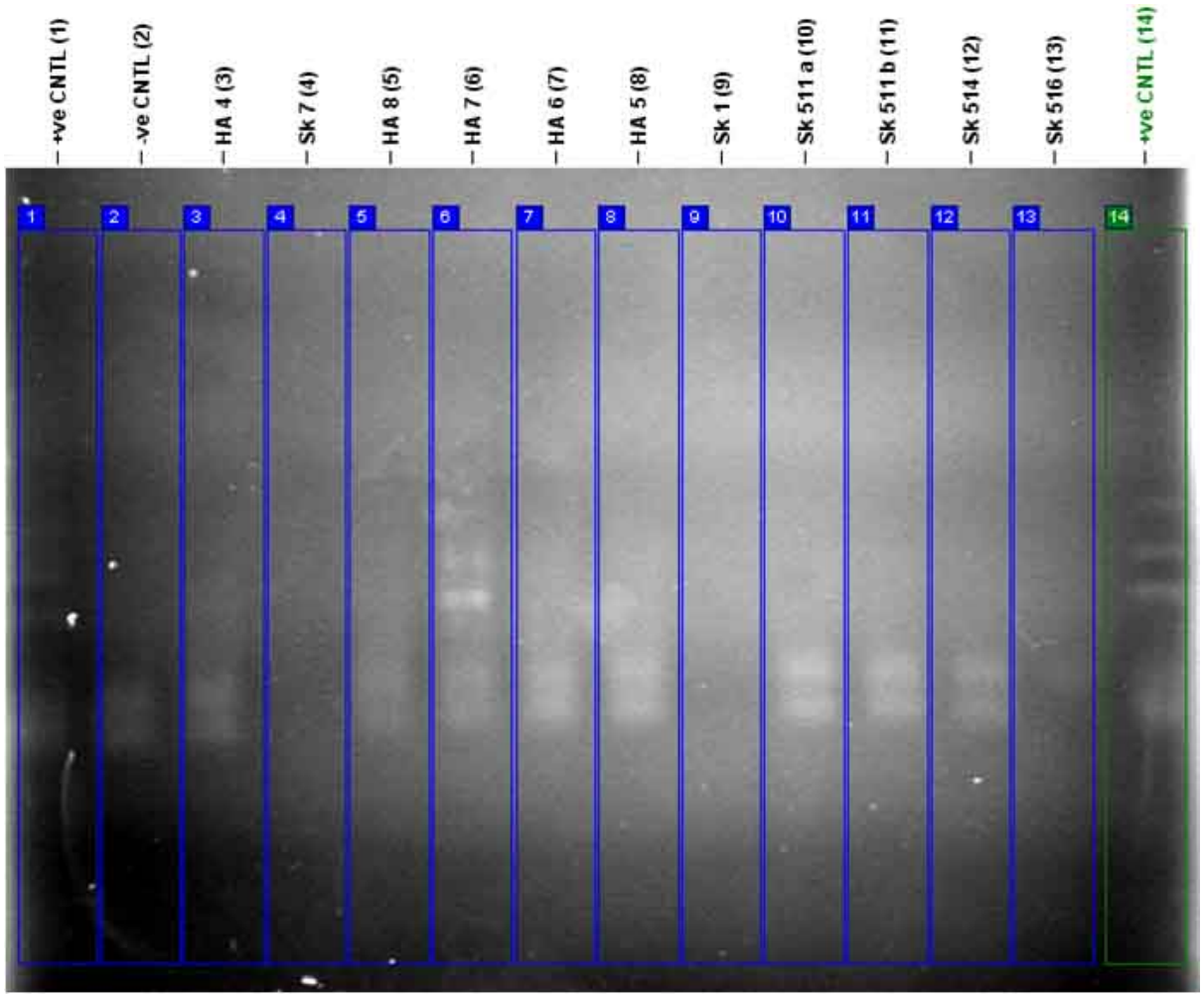


Figure 4.38: Gerstenberger mtDNA Multiplex Detection Assay for Human Samples 1-12; HA4, Sk7, HA8, HA7, HA6, HA5, Sk1, Sk511a, Sk511b, Sk514 & Sk516.

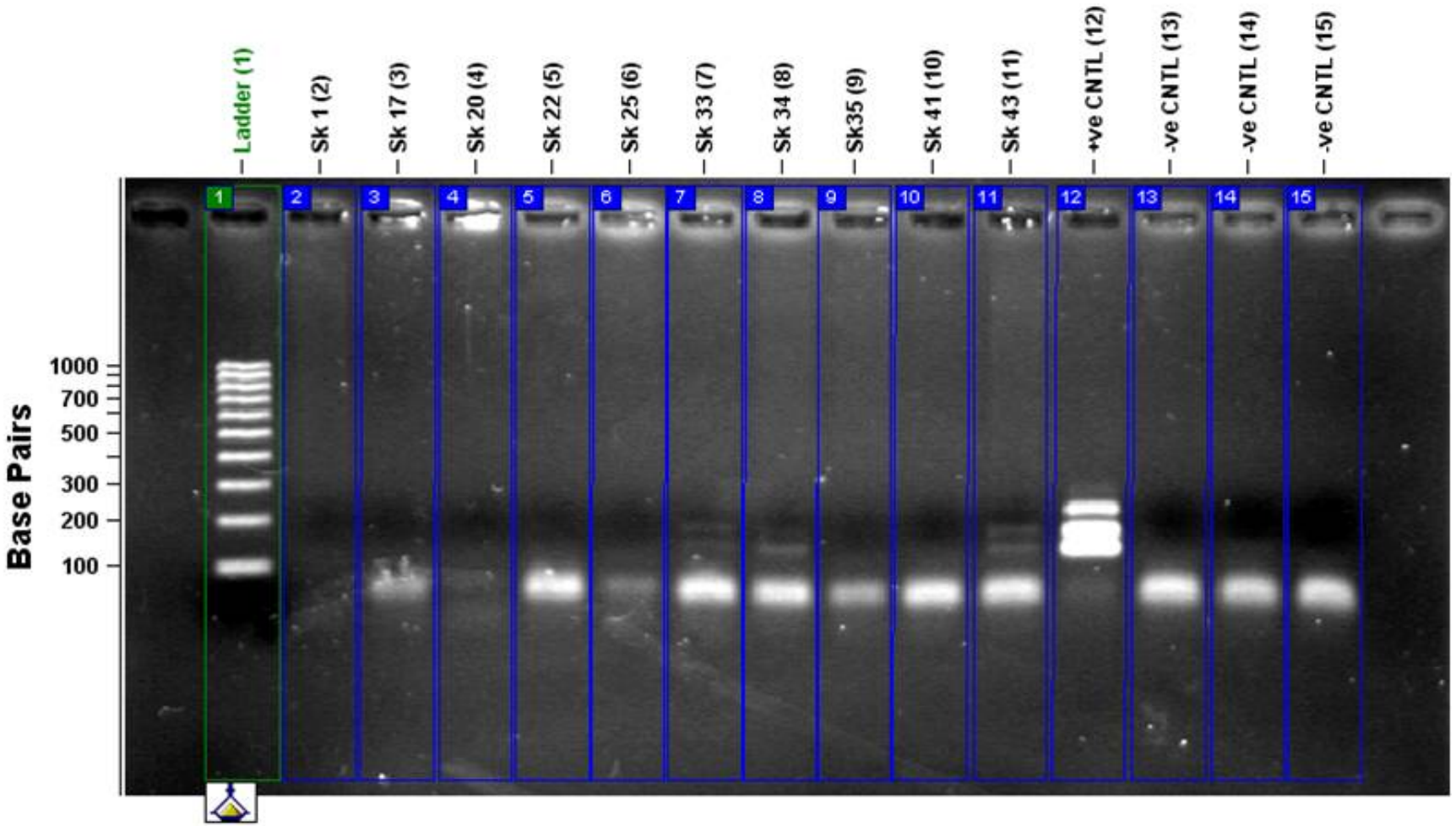


Figure 4.39: Gerstenberger mtDNA Multiplex Detection Assay for Skeletal Samples; Sk1, Sk17, Sk20, Sk22, Sk25, Sk33, Sk34, Sk35, Sk41, Sk43 & Controls for Figures 4.38-4.39.

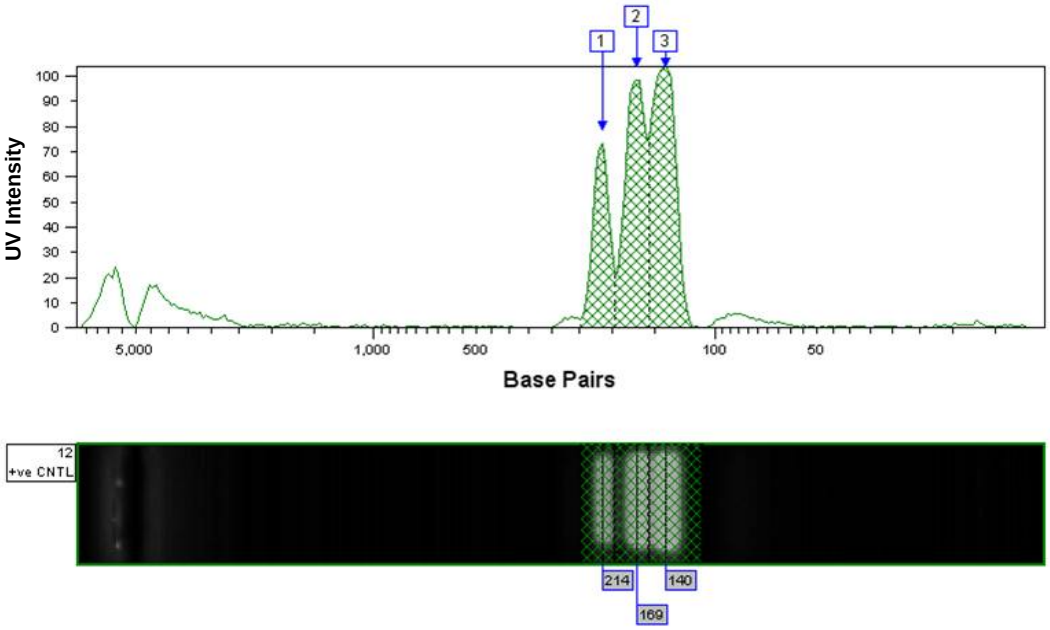


Figure 4.40a: Individual Sample Analysis of +ve control for mtDNA Multiplex by UV Band Intensity & Molecular Weight. Gel Excerpt and analysis of Control +ve from Figure 4.39.

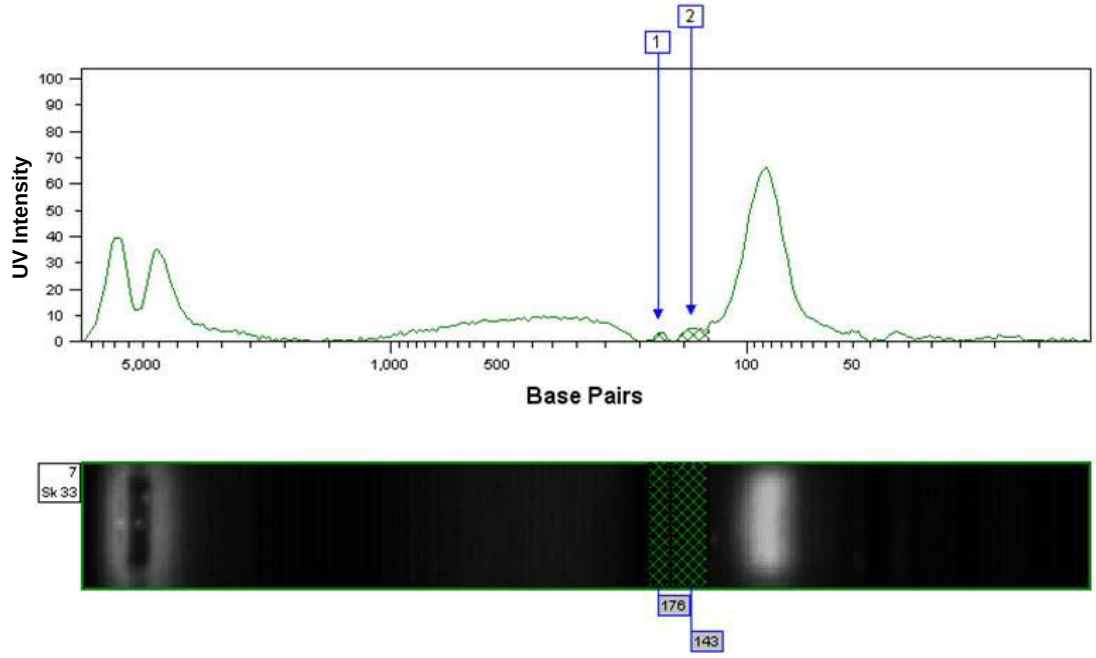


Figure 4.40b: Individual Sample Analysis of Sk33 for mtDNA Multiplex by UV Band Intensity & Molecular Weight. Gel excerpt and analysis from Figure 4.39.

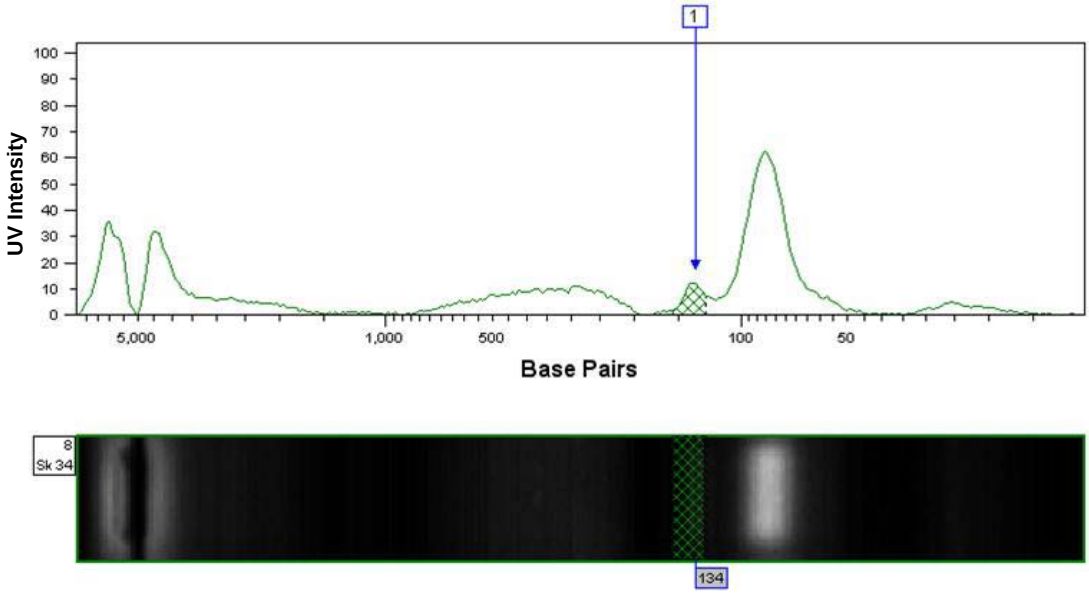


Figure 4.40c: Individual Sample Analysis of Sk34 for mtDNA Multiplex by UV Band Intensity and Molecular Weight. Gel excerpt and analysis from Figure 4.39.

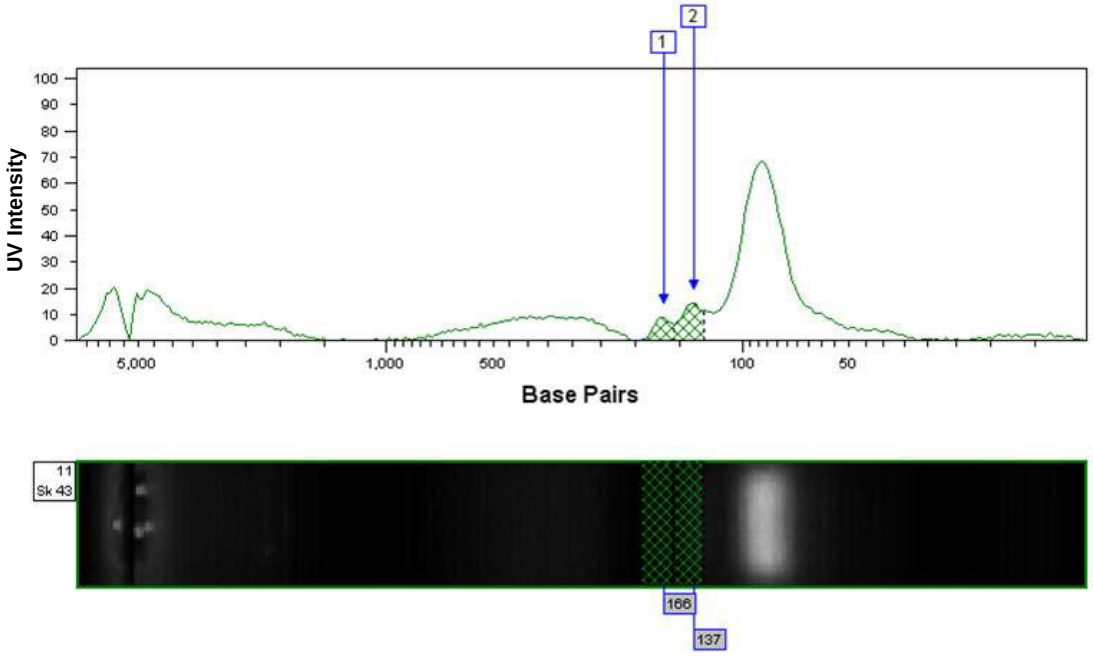


Figure 4.40d: Individual Sample Analysis of Sk43 for mtDNA Multiplex by UV Band Intensity and Molecular Weight. Gel excerpt and analysis from Figure 4.39.

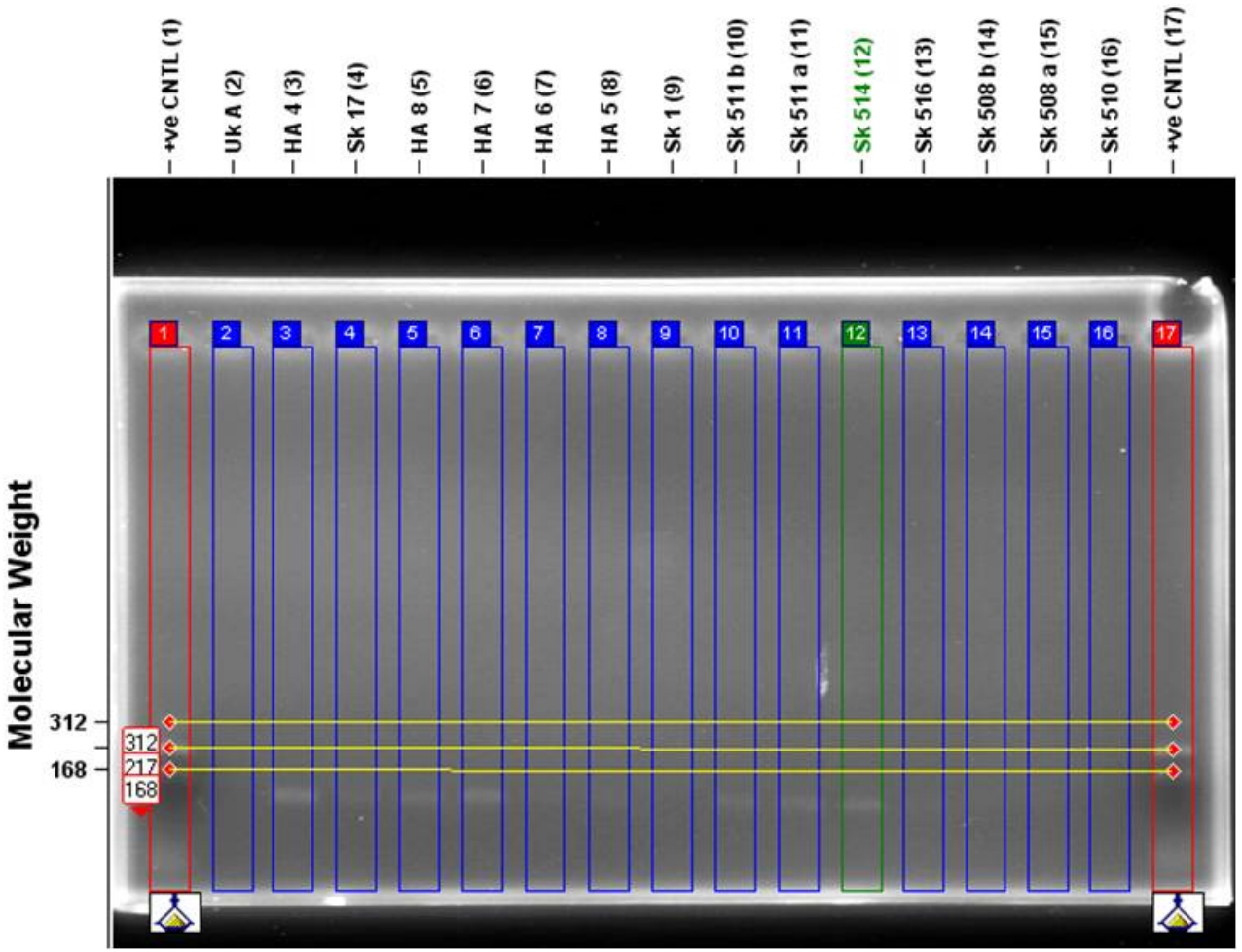


Figure 4.41: Gerstenberger mtDNA Singleplex of Human Samples 1-15; UkA, HA4, Sk17, HA8, HA7, HA6, HA5, Sk1, Sk511a, Sk511b, Sk514, Sk516, Sk508a, Sk508b & Sk510. Electrophoretic gel and sample sizing.

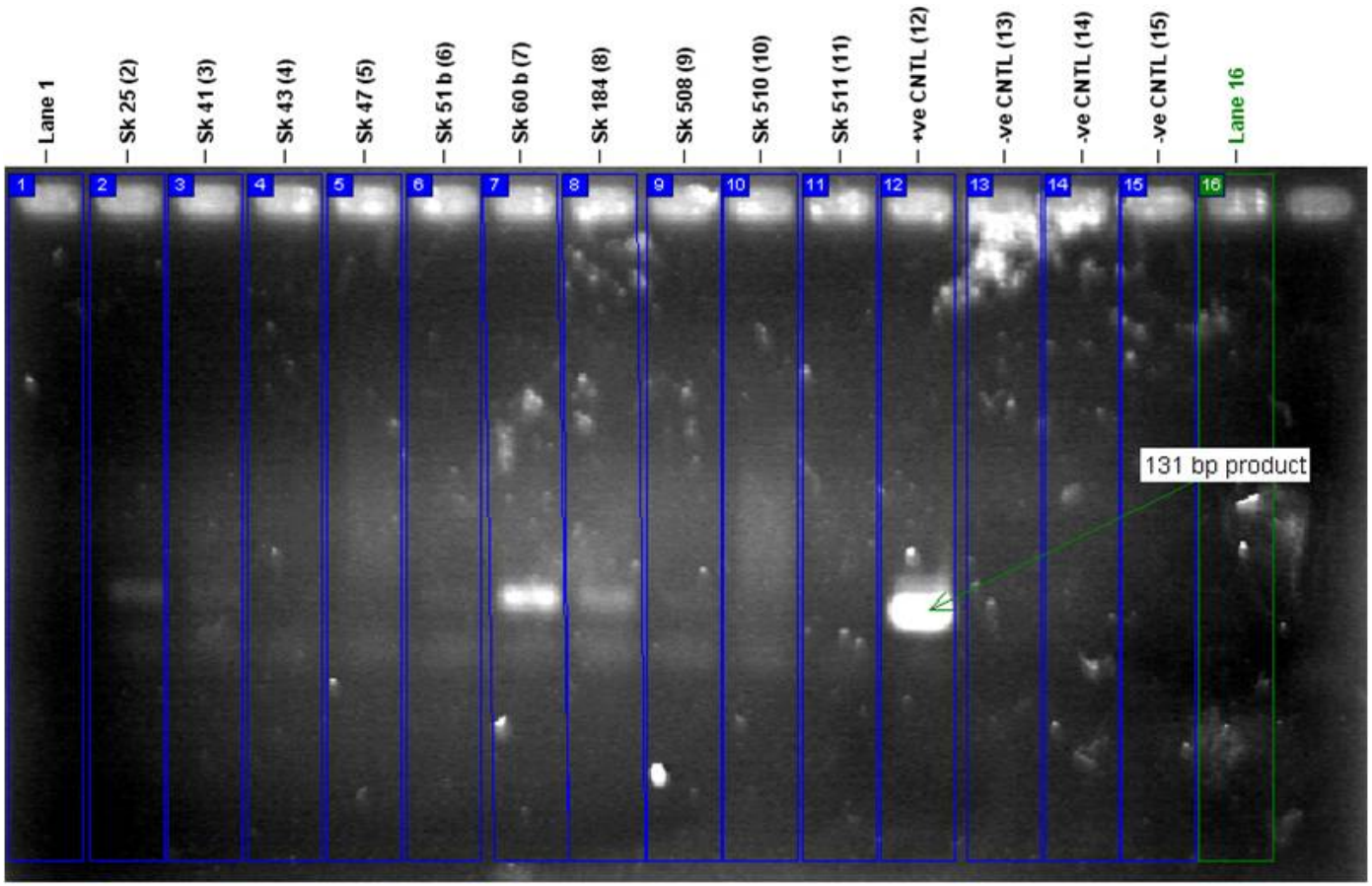


Figure 4.42: Gerstenberger mtDNA Singleplex Detection Assay for Skeletal Samples; Sk25, Sk41, Sk43, Sk47, Sk51, Sk60, Sk184, Sk508, Sk510 & Sk511.

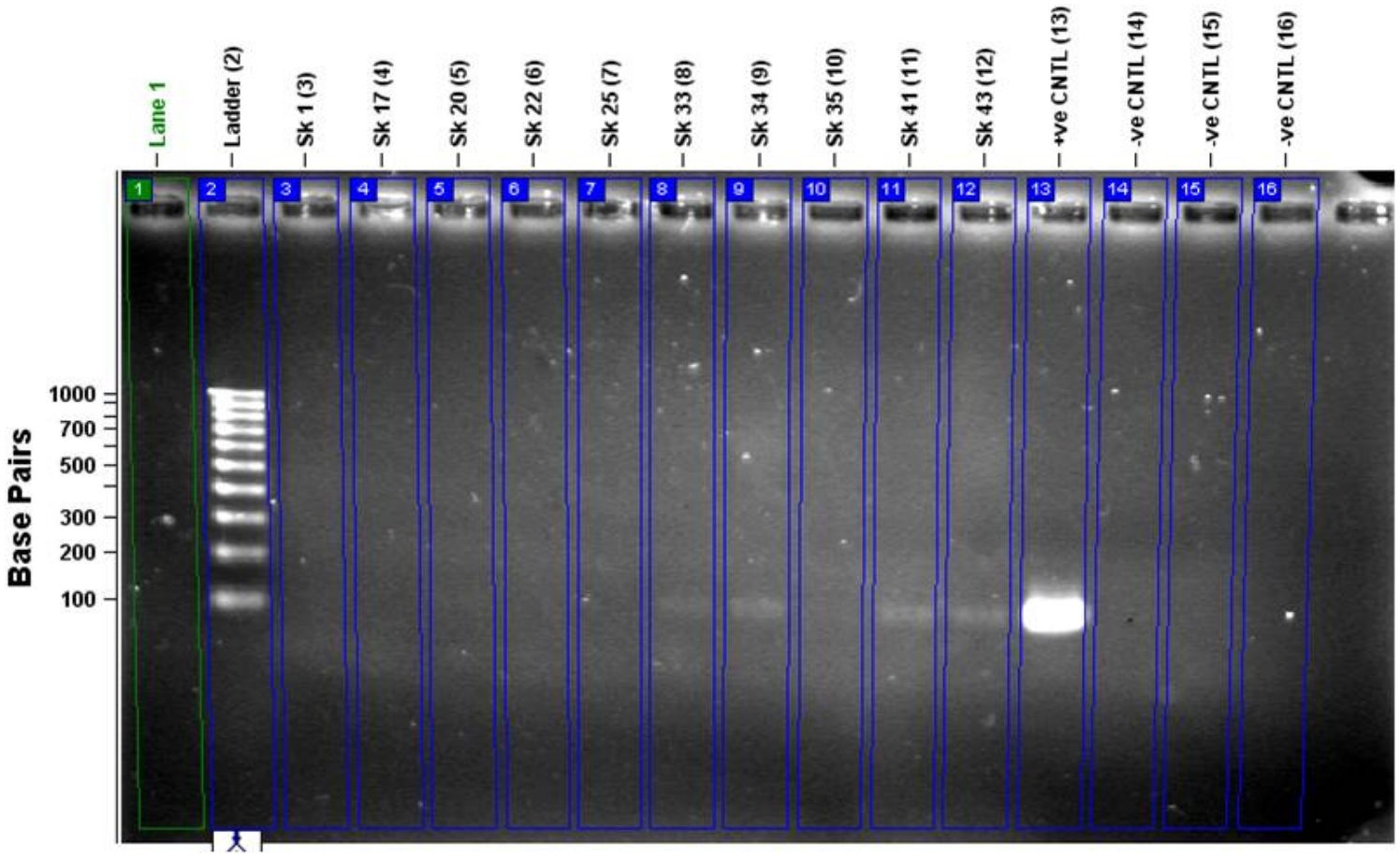


Figure 4.43:Gerstenberger mtDNA Singleplex Detection Assay for Skeletal Samples Sk1, Sk17, Sk20, Sk22, Sk25, Sk33, Sk34, Sk35, Sk41 & Sk43.

Spiking of ancient bone and teeth samples with modern DNA to determine the effect of inhibition on the amplification of ancient samples from Towyn-Y-Capel.

In order to further explore possible reasons for the lack of success in amplification of the DNA extracts from bones and teeth from Phase 1-5 extracts, samples were spiked with diluted DNA from other sources.

Using two dilutions of modern human DNA (10 ρg and 1 ρg), and DNA samples from known animal bone, samples from the site were spiked with low concentrations of human DNA and amplified with the human mitochondria multiplex primers to test for inhibition of PCR (results not shown). The Results showed PCR inhibition, as half of the amplification reactions failed to amplify the target DNA. In order to confirm these results, another more specific spiked analysis was undertaken with three different dilutions (1 ρg, 10 ρg, 100 fg) of human control DNA added to the extracted DNA solution from burial site animal bones (Figure 4.44). The results of the second spiked PCR assay confirmed the previous results, and displayed significant inhibition in over half the samples analysed, particularly with those of low modern DNA concentration.

4.5.7 Mitochondrial DNA Specific Amplification of the HV1 region in the Population samples from Towyn-Y-Capel of using the Alonso Set of Multiplex Primers.

Upon re-evaluation of Gerstenberger primer set, it was thought that some of the primers themselves may have experienced difficulty in binding to some of the templates, and that the predicted product lengths might be generally too optimistic. In addition, it was thought that primers might be mutually competitive and hence reduce amplification efficiency. In order to overcome this, and to concentrate on the singleplex component of the reaction with smaller overlapping products, the Alonso set was instead selected for future experiments as the preferred option, since it avoids these potential problems and also was able to efficiently be used for screening in multiplex reactions.

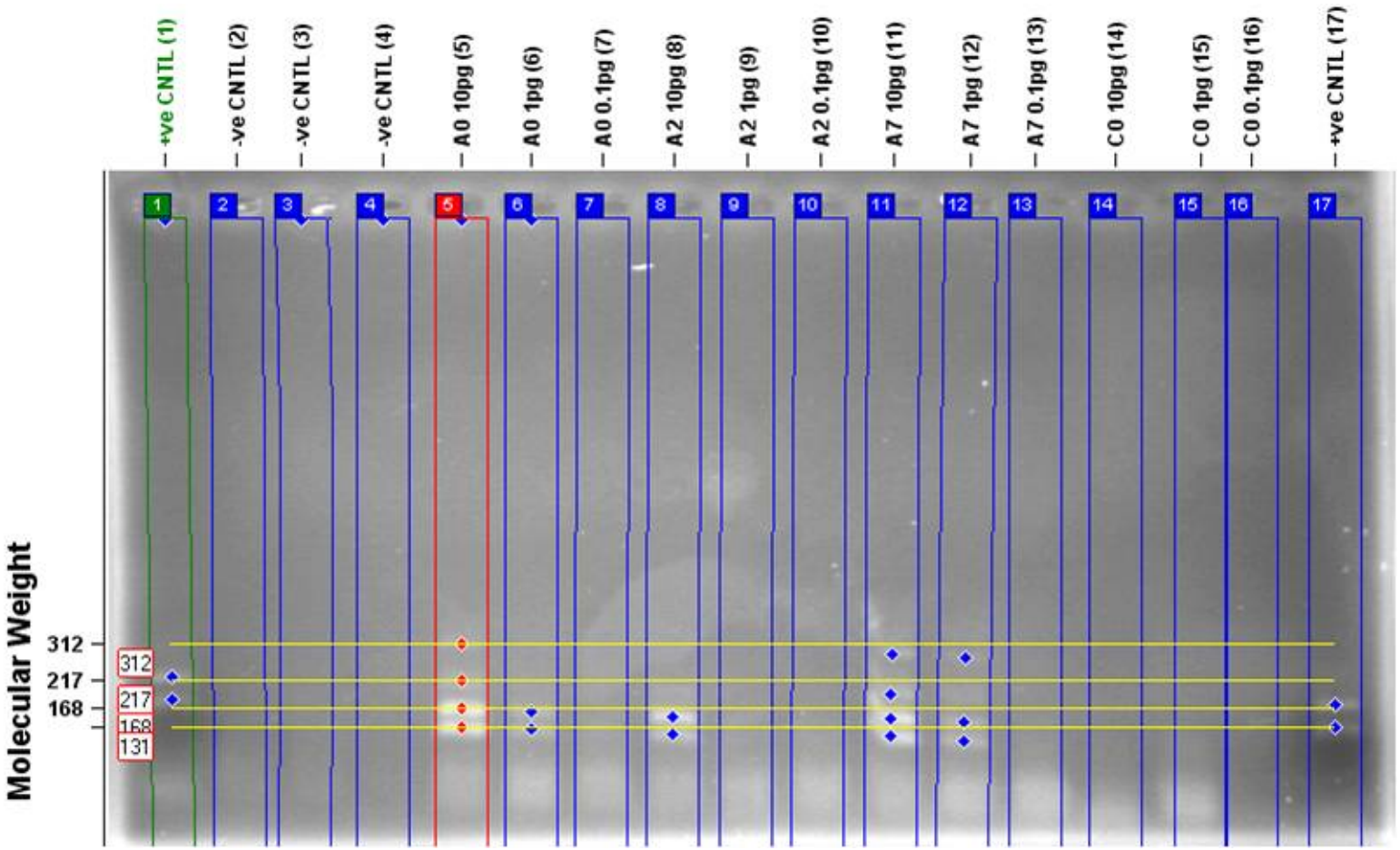


Figure 4.44: Sensitive Gerstenberger mtDNA Multiplex Inhibition Assay for Samples A0, A2, A7 & C0. The non-skeletal samples used for this assay were "spiked" with three different known concentrations (or dilutions) of modern DNA and amplified to detect possible inhibition of the PCR by constituents in the extracted material. Gel shows the electrophoretic gel of these samples overlaid by electrophoretic gel analysis, lane description and molecular weight of the multiplex bands. Results were compared against the positive controls.

Optimisation of the mitochondrial DNA HV1 specific Alonso primers set.

The Alonso primer set was selected for the same reasons as the Gerstenberger multiplex set, with the additional advantage that the primers of the Alonso set have a higher homology with a wider range of the human mtDNA HV1 haplotypes and smaller amplicon products. This yields a higher likelihood of amplification, given the expected aDNA amplicon sizes, although this advantage is at the expense of the requirement of more amplification reactions and the use of two independent multiplexes to fully screen the ancient DNA extracts. The amplification reaction was optimised as previously described for the Gerstenberger primer set (§4.3.6).

Multiplex reactions using the Alonso primer set on ancient population samples.

The Alonso multiplex is actually divided into two separate reactions. Due to the lack of success, amplification using both Multiplex 1 and 2 was only conducted on aDNA extracts from phase 1 and phase 5 and, as a screening technique, it did not yield success. At this stage, it was decided to concentrate on the two primer sets responsible the smallest products (A1F/A1R and 5F/5R) as a single product amplification.

Singleplex reactions using the Alonso primer set on ancient population samples.

Due to previous lack of amplification using DNA extracts with the Gerstenberger primers, and the failure of the screening test for the Alonso multiplex sets, it was decided to return to amplifying phase 1 and phase 5 aDNA extracts with the two smallest of the primer sets (yielding the smallest products). A1F/A1R and 5F/5R were therefore used in independent amplification reactions. In addition to this, two fluorescently labelled primers were used (one for each primer set) that would allow more sensitive detection by capillary gel electrophoresis. Negative results however were obtained for both sets of extracts, despite a higher number of cycles, smaller product length and greater homology with the mitochondrial genome. Capillary gel electrophoresis results were particularly disappointing, in that the predicted amplicons in the positive control could not be detected, despite multiple runs, and despite additional use of thin layer gel electrophoresis for detection. This was taken to indicate that the technique is not necessarily as sensitive as thin layer gel electrophoresis in the detection of small fragments of DNA (Results not shown).

4.6 Conclusion

The research has highlighted, or rather debunked recently under-considered area of aDNA research, particularly relating to the decalcification/lysis stage of the research when using small volumes, and EDTA to demineralise teeth and bone. Further in order to study the phenomenon a novel technique was discerned for rapidly determining the calcium in solution using the known principle of flocculation of the calcium-ammonium oxalate complex and UV/vis spectrometry at 400 nm.

A novel application of the polymorphic and non-specific RAPD-PCR for aDNA templates is also described clearly showing some advantages over other "specific" techniques when looking at the aDNA, particularly in ascertaining the large component of non-endogenous DNA present in ancient calcified tissues, particularly from the Towyn-Y-Capel site. The technique also shows great weakness, particularly when looking at the endogenous DNA, due to the same contamination described. This was also due to the great scarcity and variability of the amplification caused by different DNA template concentrations that is clearly shown in the variation of the human RAPD under different volumes.

With regards to ascertaining genetic information, namely mtDNA variation in the HV1 and HV2 regions from the Towyn-Y-Capel population, early success at the UCLan laboratories were met with later failure despite the use of numerous extraction, amplification techniques and assays with validated and published primers. The use of DNA spiking showed some inhibition by the ancient bone itself but was unlikely the cause for such gross levels of non-amplification, particularly given the earlier RAPD results. It was decided that the endogenous DNA present in the Towyn-Y-Capel samples was too degraded and scarce to be amplified successfully.

CHAPTER 5

MITOCHONDRIAL DNA SEQUENCED FROM TOWYN-Y-CAPEL TEETH

5 MITOCHONDRIAL DNA SEQUENCED FROM TOWYN-Y-CAPEL TEETH

5.1 Introduction: Independent Validation of Mitochondrial DNA Specific Amplification of DNA Extracted from Dental Tissues for Sequencing

Separate aliquots of the original separation of milled tooth were sent for analysis at the investigators expense to an independent specialist laboratory for independent validation and amplification of selection of teeth (Table 5.1).

The laboratory (paleoDNA laboratory, Lakehead University, Canada) was selected due the specialist ancient laboratory setup and separate facilities that meet conditions required for ancient DNA analysis and standards accreditation CA-P-4E (ISO/IEC 17025), P-1578 and American Society of Crime Laboratory Directors (ASCLD) compliant. Sequencing was restricted due to financial and temporal restraints to a selected sample set chosen due to gross skeletal condition (Table 2.2), gross sample and inorganic faction condition (§ 2.3.2; §2.3.5), organic faction condition as determined by amino acid racemization (§ 3.41) and prior success in genetic amplification (§ 4.5).

5.1.1 Methods: Extraction and Purification Procedure used for Sequenced Dental Tissues

The DNA was extracted using a protocol modified from Lorielle et al., 2007. In a 2.0 ml tube with 1.5 mls of 0.5M EDTA, 75 µl 20% lauryl sarcosinate and 40 µl of proteinase K (20 mg/ml) was added approximately 0.2 mg of powdered material for each sample. This was then vortexed, sealed with parafilm and incubated at 56^{o}C at 1000 rpm overnight to decalcify. Phenol chloroform: isoamyl alcohol purification was used, where each extract was separated into two aliquots of 800 µl to which 200 µl phenol and 200 µl chloroform: isoamyl alcohol (24:1 v/v) was added, and the sample was vortexed to achieve a milky emulsion and centrifuged for 5 min at 16000 rcf. The upper aqueous layer was removed and another 200 µl Phenol and 200 µl chloroform: isoamyl alcohol (24:1 v/v) was added, vortexed and centrifuged again. The upper aqueous layer was again removed and 400 µl of chloroform: isoamyl alcohol (24:1 v/v) was added vortexed and centrifuged again, of which the upper aqueous layer was removed and placed in a 2 ml tube.

This extract was separated into 3 aliquots (of approximately 500 µl each) and concentrated using ethanol precipitation. 50 µl of 3 M sodium acetate was added to each extract, and votexed for 1 min, after which a value of 1200 µl of ice-cold ethanol was added and the tube is placed in freezer (-20^{o}C) overnight. Tubes were then briefly thawed, and centrifuged for 5 min at 16000 rcf.

The supernatant was discarded and the precipitate was then washed by resuspension in 500 µl of ice cold ethanol. This was then vortexed and centrifuged again for 10 min at 16000 rcf, and the supernatant was discarded and the pellet left to dry for 1 hour. The pellet was resuspended in 100 µl Tris EDTA (TE) buffer at 37^{o}C at 350 rcf for 15 min for column purification. A Micro Bio-Spin® 30 Column was used to purify sample according to manufacturer's instructions. The biospin column was placed in 1.5 ml tube into which the extraction solution was added and centrifuged at 1000 rcf for 4 min. The column is then discarded and the flow through kept for PCR amplification

5.1.2 Method: Amplification Procedure used for Sequenced Dental Tissues

Platinum *Taq* DNA Polymerase was used as follows: in a 25 µl reaction volume 0.1 µl of Platinum *Taq*, 2.5 µl 10 X PCR Buffer, 0.5 µl 10 mM dNTP Mixture,1.0 µl 50 mM $MgCl_2$, 0.5 µl Primer mix (10 µM of each Primer) and 18.4 µl PCR clean water to which was added 2 µl of templateDNA. Sequence reactions were all singleplex using the primers shown in table 5.2 specific for the human mtDNA hypervariable region 1 (HV1 base pairs 16024-16365 rCRS, Table 5.3). The PCR programme was 95^{o}C for 2 min; 50 cycles of 95^{o}C for 30 min, 56^{o}C for 1 min, 72^{o}C for 2 min. PCR program is notable for the high number of cycles. Prior to amplification a dilution series was completed on each sample. Amplification was performed on an Eppendorf Mastercycler 5333.

Table 5.1: Anthropological Details on the Sequenced Skeletal Teeth

Sk#	Sex[a]	Skeletal Condition[a]	Skeletal Preservation [a]	Age[a] (years)	Tooth Type[b]	Tooth Condition[c]	Weight (mg)
Sk21	M	Mod	Mod	Adult	RM^3	2	207.80
Sk33	F	Mod	Mod	20-30	RM_3	2	194.00
Sk34	M	Mod	Good	35-45	LM_3	2.6	200.95
Sk35	M	Good	Good	15-25	LM_2	1	243.50
Sk41	M	Mod	Good	30-45	RM_2	2	264.60
Sk51	M	Mod	Good	30-49	LM_3	3	209.60
Sk52	F?	Poor	Mod	25-45	RM_2	2.4	250.70
Sk56	M	Good	Good	50-60	LM_3	N/A	220.60
Sk57	?	Mod	Mod	25-35	RM_2	N/A	195.60
Sk60	F	Good	Good	35-45	LM_1	N/A	196.31
CNTL[d]	N/D	Excellent	Excellent	N/D	C	1	360.8[d]

Breakdown of the anthropological details of source skeletons and weight of powdered tooth tissues used for successful aDNA analysis. [a]Estimates utilizing qualitative data from corresponding osteological investigation, see Table #2.2; [b]Tooth position and type used for the analysis, see § 1.331 for more detail regarding nomenclature; [c]Gross Preservation Index (GPI) of the dental sample itself (§ 2.42), ranging from 1 -4 (1 -Poor to 4- Excellent); [d]Used for contamination control; [e]Not all sample used.

Table 5.2: HV1 mtDNA PaleoDNA Sequencing Primers

Designation	Sequence	Tm* (^oC)	Reference
HV1B_fwd	5'-CCC ATG CTT ACA AGC AAG TA-3'	56	Kolman et al., 2000
HV1B_rev	5'-TGA TTT CAC GGA GGA TGG TG-3'	58	Vigilant et al., 1989
HV1A_fwd	5'-TTA ACT CCA CCA TTA GCA CC-3'	56	Pfeiffer et al., 2001
HV1A_rev	5'-TGG CTT TGG AGT TGC AGT TG-3'	58	Tully et al., 2000

Designation, sequence, melting temperature and referenced source of Primers used for amplification and sequencing of HV1 region in aDNA samples.*Basic Salt Adjusted Oligonucleotide Melting Temperature (Tm) calculated using Oligonucleotide Properties Calculator[1].

Table 5.3: HV1 mtDNA Sequencing Amplicon Details

Primer Group	Designation	rCRS Position	Primer Length	Amplicon Length	Sequence Length	Sequence rCRS Range
HV1-D	HV1A_rev	16193>16212	20 bp	229 bp	189 bp	16213>16402
	HV1 A_fwd	16403>16422	20 bp			
HV1-E	HV1B_rev	15972>15991	20 bp	288 bp	258 bp	15992>16240
	HV1B_fwd	16241>16260	20 bp			
HV1 -F	HV1B_rev	15972>15991	20 bp	450 bp	410 bp	15992>16402
	HV1 A_fwd	16403>16422	20 bp			

[1] www.basic.nwu.edu/biotools/oligocalc.html

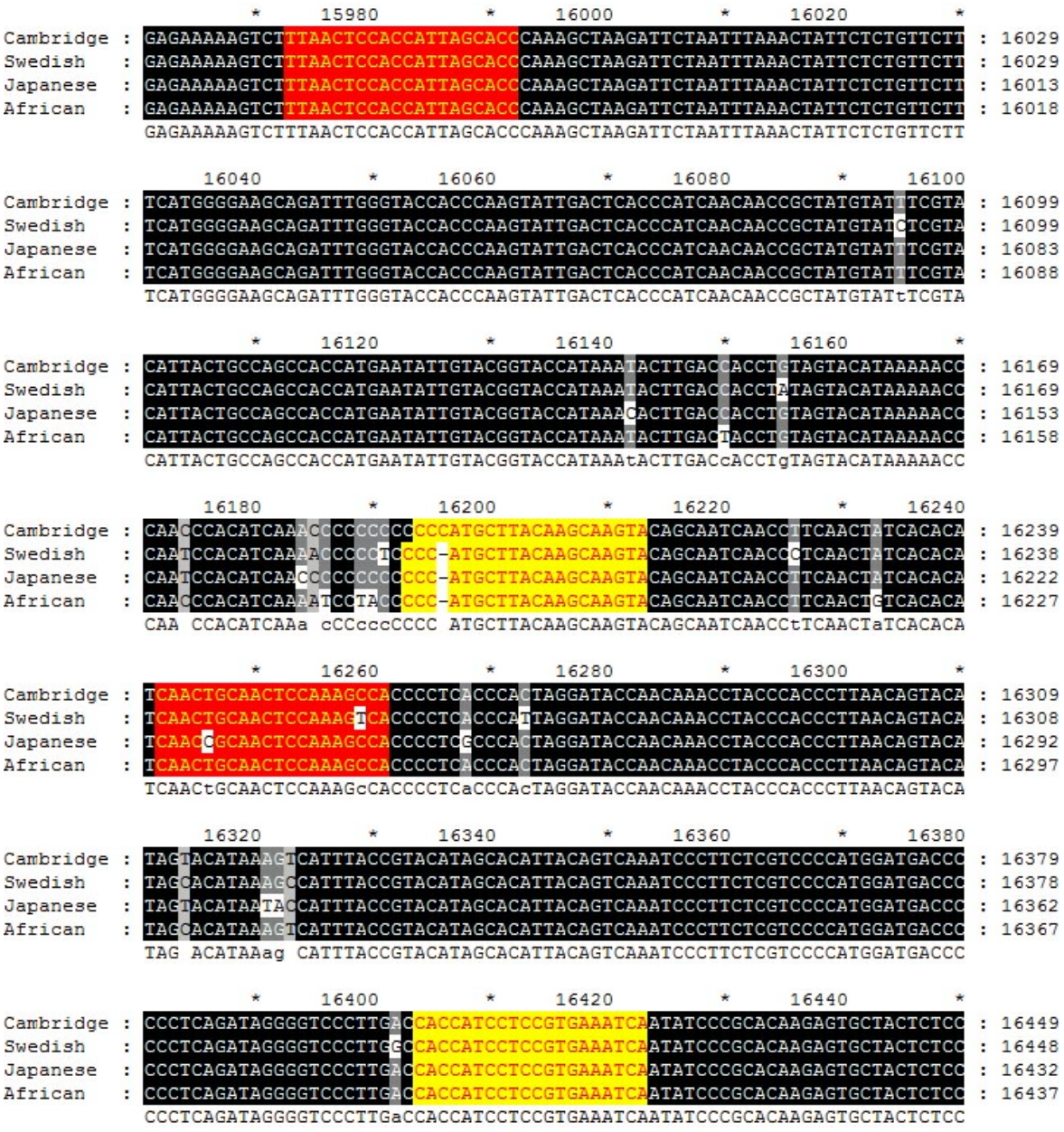


Figure 5.1: HV1 Sequencing Primers Relative to rCRS Position on Alignments of 4 Reference Mitochondrial Sequences.

Primer pairs are designated by background colour/text as HV1-A (yellow on red), HV1-B (red on yellow). Alignments constructed using GeneDoc, version 2.6.002 (Nickolas *et al.*, 1997), against reference sequences as in figure 4.1.

5.1.3 Method: Direct Sequencing

The actual sequence of PCR products can be obtained from the purified PCR product using a technique called big Dye sequencing. Successfully, and repeatedly amplified sequences were purified and sequenced using ABI Big Dye Chemistry v3.1 and run on the ABI 3100 Genetic Analyzer on factory settings as recommended by the manufacturers (Applied Biosystems).

5.1.4 Method Sequence Compilation, Alignment and Verification

Sequence analysis was performed using DNAstar Lasergene 8.0 SeqMan Pro, where sequences were assembled and aligned with MegAlign against other samples comparable the control sequence, the rCRS (NC001807) to determine mitochondrion Haplotype (Ht) based on hypervariable region 1.

Difficult templates were analysed manually with Applied Biosystems Sequencing analysis software v5.2 .Assembled sequences were compared against available mtDNA databases. Whole sequences were investigated through the over 3900 mtDNA sequences of the NCBI public domain Database[2]. Additionally single nucleotide deviations (transitions, deletions and additions) were compared against other populations such as the open access FBI mtDNA database[3], EMPOP[4] and the genographical project (Behar et al., 2007) in order to investigate the mostly commonly associated Hts and Haplogroups (Hgs) with the polymorphisms found in the population at the HV1 region. This is determined by investigating large population datasets that may contain similar polymorphisms in the hypervariable region in modern human sequences. Although absolute determination of Hgs cannot be ascertained completely, in most cases, with the hypervariable region data alone, the hypervariable region allows us to see the most diverse region of the mitochondrial genome, and subclade classifications of the major Hgs can be determined or even family groups.

[2] http://www.ncbi.nlm.nih.gov/
[3] www.ianlogan.co.uk/haplotype/finder.htm
[4] www.EMPOP.org

5.2 Sequencing Results and Discussion

Direct sequencing can be problematic with respect to aDNA, as contaminating sequences may be difficult to distinguish from endogenous DNA which is normally present in a damaged condition. However, modern technology can allow this to be overcome to some degree by the use of multiple sequencing runs and careful analysis of the resultant sequence profiles. Multiple direct sequences were therefore performed for each target region (HV1 A and HV1 B) for each tooth sample. This approach was adopted to reconstruct the sequences as faithfully as possible, in order to identify sequences that might arise from any anomalous amplification, non-representative sequences and molecular misinformation caused by incorrect base incorporation by the *Taq* Polymerase. The latter can occur for a variety of reasons, including base excision and base deamination, but primarily it is more usually due to the poor condition of the aDNA template that the DNA polymerase is attempting to copy.

Multiple sequencing of PCR amplicons can potentially identify this. In addition to detecting and resolving amplification issues, multiple sequencing can be used to detect gross contamination, where more than one template may be present. Hypervariable region 1 sequences from aDNA samples were constructed using direct sequencing of the overlapping sequences amplified using HV1 A and HV1 B primer pairs independently. A total of 10 sequencing runs were carried out per sample, using both forward and reverse primers, and normally five sequence runs were carried out on each amplification product. Not all samples could be amplified, and of those that were amplifiable, not all samples resulted in an entire sequence, as can be seen in table 5.5. Compiled sequences and alignments can be seen in Appendix 5.1.

Generally, the degree of success in the amplification and sequencing of the ten teeth samples selected was better than expected, as the previous experimentation into the quality of the ancient samples indicated that the samples were extremely difficult to amplify successfully and reproducibly. Further investigation into the sequences themselves however is helpful in clarifying the situation with regards to the nature and origin of these sequences. It is thought that the success, compared to the previous quality results, may have been due to the extreme amplification profile used of 50 cycles or more, which was not used in previous PCR experiments in the laboratories at UCLan. Experiments which amplify DNA through more than 40-50 cycles requires specialist facilities in order to prevent spurious contamination, such as positive air pressure and positive displacement pipettes, dustless laboratories and UV lighting.

Sequences were analysed using DNAstar suite. The sequences examined by this software themselves varied in quality, even within the individual sequence run. The sequences were normally of reasonable quality at the primer end but showed some deterioration in condition further into the run. Sequences were run from each primer of the primer pair, which facilitated the reconstruction of the whole sequence, and HV1A and HV1B sequences were then aligned.

The variable quality and length of many of the sequence runs required the use of the revised Cambridge reference sequence as a framework to align and collate the data to produce a consensus HV1 sequence for each of the samples investigated. Unusually, much of the individual sequence data also showed unique polymorphisms, which indicated that the PCR was mis-amplifying bases or that there may have been contaminating sequences in the sample.

The rarity of the DNA (as the general and individual area peak height) and the occasional deletions support this view. The problem was further compounded when compiling consensus sequences where the data was either dropped as being irrelevant or placed as a changeable mutation. In order to alleviate this situation without loss of information, a table of the different polymorphisms, whether common mutations or not, was compiled, and the nature of the polymorphism was identified (Table 5.8).

The objective of this analysis was to distinguish true or important mutations from possible contaminations. There may be more than one sequence present in each aDNA sample, and the polymorphisms could belong to both or either, and occur at a random distribution that cannot be determined or may arise from a side effect of the aDNA itself: base modification can give rise to incorrect base replacement in the amplification process or even base pair dropouts. This is particularly relevant where some polymorphisms are more likely than others to drop out or modify than others. Great caution was therefore required in the compilation and acceptance of amplified aDNA sequences compiled in this way.

Visual inspection and confirmation of the sequences using SeqMan for deviations and anomalies was required, particularly in cases where the sensitivity or the appropriate selection of base pairs to peak was inappropriately accomplished by the program itself. Due to the irregular nature of aDNA, and the small amount of template, there are often cases where the DNA has been poorly amplified or where background non-specific Big Dye binding obscures the individual peaks or peak areas, and sometimes caution requires the exclusion of such data.

Overlapping sequences of HV 1, both A (15972>16260) and B (16193>16422) were assembled into contigs for HV1 region reconstruction of the consensus sequence. This was carried out in order to verify that the sequences were true representations of the endogenous ancient human DNA. The variable condition of sequences, primarily due to poor amplification and sequencing, was evidenced in the limited peak height and sequential peak deterioration further from the vector sequence, and was in line with the expected aDNA sequencing behaviour, as the longer sequences are harder to amplify and sequence due to the decreasing proportion of the longer sequences in the original DNA template. Other deleterious conditions included occasional base pair dropout (shown as a deletion) and molecular misinformation arising from incorporation of incorrect bases which can be detected as an unusual and random substitution or deletion in sequences where normally these are rare or unrecorded.

Such polymorphisms must be investigated carefully, and must often be excluded as anomalous data caused by the mis-amplification of aDNA. A brief synopsis of common mtDNA HV1 polymorphisms spanning base pairs from 16000-16420 (relative to rCRS), was used for identification of Ht and the sequence results, as preliminarily ascertained and compiled are given in table 5.6.

Upon further analysis of the preliminary sequence data and assembled sequences, some discrepancies were found in relation to the base calling and the minute peak differences. These are important in understanding the nature of the miscoding lesions within the sequences. Therefore, sequences were reconstructed using the rCRS (Hg H) as a framework, to compensate for intra sequence degradation that occurs principally at the overlapping areas. Sequences were re-verified along their entire sequence and in particular, point mutations were identified and classified.

Table 5.4: Sequence Run Success and Assembly.								
Sk#[1]	**AB1 Files[2]**		**Fasta Files[3]**	**Run Success[4]**	**Length Assembly[5]**	**rCRS Positions[6]**	**Length Success[7]**	**Total Success[8]**
	HV1a	**HV1b**						
Sk21	5	5	10	100%	450 bp	15970-16420	100%	100%
Sk33	5	5	7	70%	450 bp	15970-16420	100%	70%
Sk34	4	5	9	100%	450 bp	15970-16420	100%	100%
Sk35	5	5	4	40%	450 bp	15970-16420	100%	40%
Sk41	5	5	9	90%	450 bp	15970-16420	100%	90%
Sk51	0	12	6	50%	160 bp	16190-16367	35.56%	18%
Sk52	4	5	9	100%	450 bp	15970-16420	100%	100%
Sk56	4	5	8	88.89%	396 bp	15971-16367	88%	78%
Sk57	0	11	6	54.55%	131 bp	16191-16322	29.1%	16%
Sk60	2	4	N/A	N/A	N/A	N/A	N/A	N/A

The table above shows the number of sequence runs for each skeleton and target (HV1a or b) as ABI files, and of these, how many successful sequences were recovered (as Fasta files) how long and what part of the HV1 sequence was successfully sequenced. [1]Skeletal Sample Designation, [2] Proprietary Applied Biosystems Electropherogram files for both primer sets (HV1a & HV1b) including number of forward and reverse sequence runs for each primer set, [3] Total number FASTA format files of interpreted data from AB1 files, [4] Percentage of sequence runs that yielded sequences, [5] Final length of HV1 contig assembled from all sequence runs, [6] Sequenced area relative to rCRS position, [7] Percentage of recovered sequence relative to sequenced area. [8] Combined sequence run success and total length recovered.

Figure 5.2 (Next Page) Screenshot of DNAstar Seqman Sequence Assembly of Sample Sk21A.
Original sequence assembly view of the multiple sequence runs compiled into readable sequences and compared against for direct conformation and verifiability of base calls and detection of possible VPT's for the purpose of determination from multiple experimental runs.

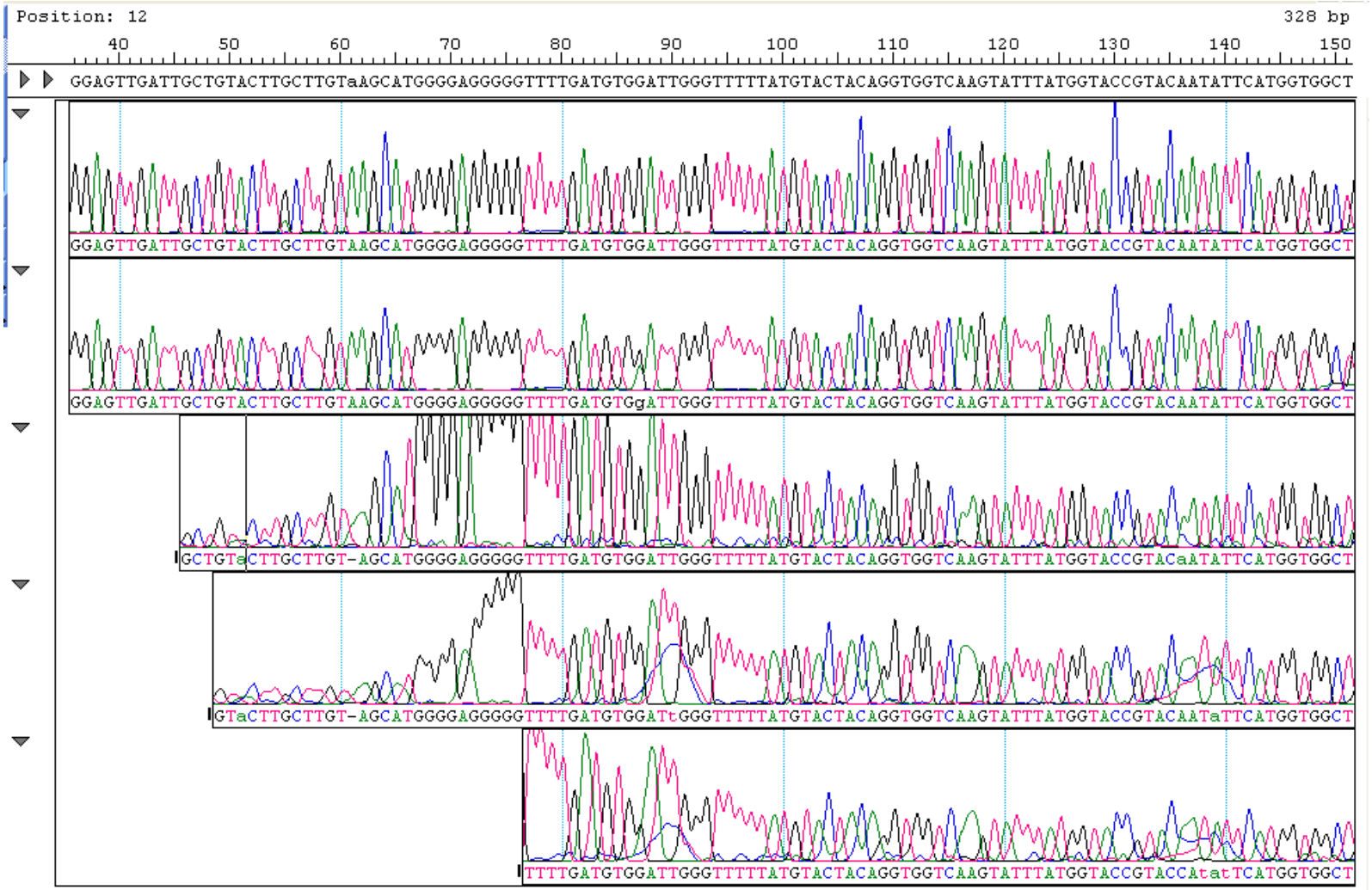

Position: 12
328 bp
40
50
60
70
80
90
100
110
120
130
140
150
GGAGTTGATTGCTGTACTTGCTTGTaAGCATGGGGAGGGGGTTTTGATGTGGATTGGGTTTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATATTCATGGTGGCT
GGAGTTGATTGCTGTACTTGCTTGTAAGCATGGGGAGGGGGTTTTGATGTGGATTGGGTTTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATATTCATGGTGGCT
GGAGTTGATTGCTGTACTTGCTTGTAAGCATGGGGAGGGGGTTTTGATGTGgATTGGGTTTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATATTCATGGTGGCT
GCTGTaCTTGCTTGT-AGCATGGGGAGGGGGTTTTGATGTGGATTGGGTTTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACaATATTCATGGTGGCT
GTaCTTGCTTGT-AGCATGGGGAGGGGGTTTTGATGTGGATtGGGTTTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACAATaTTCATGGTGGCT
TTTTGATGTGGATTGGGTTTTTATGTACTACAGGTGGTCAAGTATTTATGGTACCGTACCAtatTCATGGTGGCT

Table 5.5: Common HV1 Polymorphisms of Towyn-Y-Capel Sequenced Samples.

Sample	16021	16049	16052	16130	16182	16192	16212	16218	16222	16224	16225	16228	16245	16250	16252	16259	16260	16270	16278	16289	16294	16295	16296	16311	16320	16329	16330	16342	16343	16354	16356
rCRS	C	G	C	C	C	C	A	C	C	T	C	C	C	C	A	C	C	C	C	A	C	C	C	T	C	G	T	T	A	C	T
21	C	G	Y	C	C	C	A	C	T	C	C	C	C	C	A	C	C	T	C	A	C	C	C	Y	C	G	T	T	A	C	T
33	Y	G	C	C	C	C	G	C	T	C	C	C	C	C	A	C	C	T	C	A	C	C	C	Y	C	G	T	T	A	C	T
34	C	R	C	M	T	T	A	C	C	T	C	C	C	C	R	C	C	C	Y	A	C	C	C	Y	C	G	T	T	A	C	T
35	C	G	C	C	C	C	A	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	Y	Y	G	T	T	A	C	T
41	C	G	C	C	C	C	A	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	T	C	G	T	T	A	C	T
51	N	N	N	N	N	C	A	C	T	T	T	T	T	C	A	C	C	C	C	A	C	Y	C	T	C	R	T	C	R	C	C
52	C	G	C	C	C	C	A	C	C	T	C	C	C	C	A	C	C	C	C	A	C	C	C	T	C	G	T	T	A	C	T
56	C	G	C	C	C	C	A	C	C	C	C	C	C	C	A	C	C	C	C	A	Y	C	Y	Y	Y	G	T	T	A	C	T
57	N	N	N	N	N	C	A	Y	C	T	C	Y	C	Y	A	Y	Y	C	C	M	C	C	C	T	C	G	T	T	A	T	T
60	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	T	C	G	K	N	N	N	N

The table above outlines preliminary analysis of recovered sequences compared to the rCRS. Table does not address all ambiguous base calls, or differentiate between those undetermined base calls. rCRS=revised Cambridge Reference Sequence (Andrews et al., 1999; Anderson et al., 1981); Y= undetermined pyrimidine (C or T); R= undetermined purine (A or G); M= undetermined amino base (A or C); K= undetermined keto base (G or T); N= no detection in this area

5.2.1 Sequence Compilation and Analysis

Sequences were compiled using the Seqman programme, which is part of the suite of DNA star Lasergene. Sequences from the ABI 3130 electropherograms were analysed using this programme and assembled using the default criteria, with low quality end trimming along with the marked reference sequence rCRS. The marked reference sequence was necessary as a template to position and assemble the fragmented forward and reverse amplicons into one cohesive whole. Additionally, the use of the reference sequence facilitated the binding of the two overlapping sequence regions HV1 A and HV1 B to produce a cohesive and easily comparable region of approximately 450 base pairs.

Interpretation of the sequences provided needs to be undertaken individually. All base calls are made upon positions and variations from the revised Cambridge Reference Sequence (rCRS). In terms of notation, base changes and positions are given in the following order: the rCRS base, position, and base found at that location. For example, a single base mutation at position 16222, where the base C in the rCRS is found to be T in the analysed sequence, is given in this shorthand notation as C16222T.

Assembled sequences were further edited to correct and observe any miscellaneous base calling through a thorough analysis of the electropherograms (using the Seqman program, Figure 5.2) and comparison with the other sequence electropherograms, to construct a consensus sequence for that particular individual. Unfortunately, miscellaneous cases of contradicting base calling were encountered, particularly at the end of sequences (commonly a base fallout was shown as a deletion) but also in known polymorphic areas.

Variable base calling at these polymorphic areas fell into two distinct classes, and for the purposes of this chapter these will be described as Variable Polymorphic Types 1 and 2 (VPT 1 & 2) (Table 5.7). VPT 1 refers to separate contiguous sequences that show different bases at the same position, while VPT 2 is characterised by peaks corresponding to two bases present at the one nucleotide position within a single sequence where, normally, one peak is more significant than the other, or in some rare

cases, both are the same height. The predominant peak characteristic of the VPT 2 class of polymorphism is normally of reduced size and fluorescent intensity than would otherwise be expected due to normal background (Tully et al., 2000).

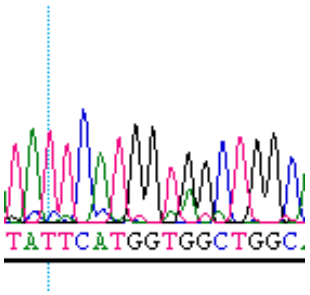
There are four main reasons reported for this anomaly:
1) The sequence is contaminated, either by one or more DNA sources,
2) Incorrect base calling by Taq polymerase, primarily caused by a modified base at that position (miscoding lesion) such as a pyrimidine that has been deaminated due to genetic diagenesis (§1.3.4), and which in turn leads to spurious base calling by the polymerase.
3) Incidence of heteroplasmic mitochondrial genomes due to multiple copies of different mtDNAs.
4) Incidence of microheteroplasmic mitochondrial genomes with multiple variations in polymorphic sites in the mtDNA.

Direct sequencing of amplified DNA products from aDNA samples provides limited information as to the specific nature of the anomalous base calls. This can be clarified by cloning of the amplicons, although this technique may itself introduce further artefacts into the sequencing reaction. Repeatability is the main key in ancient DNA research to confirm results, and hence sequences that are variable when repeated (such as class VPT 1) are more likely to arise from experimental error and/or contamination.

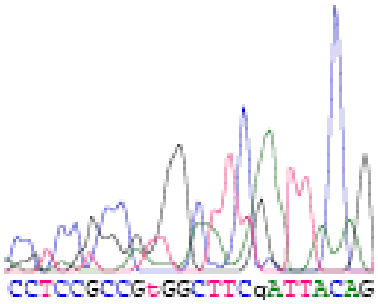
This does not exclude VPT 2 from being also possibly due to contamination. It is possible to exclude gross contamination of samples at source or in the laboratory, through the institution of preventative measures as described previously in chapters 1, 2 and 3, it is most likely that a single site polymorphic variation exists in the endogenous mitochondrial genome. This can arise through one of two different mechanisms in modern individuals, namely heteroplasmy and microheteroplasmy. It is generally accepted that heteroplasmy in humans is a rare event although to date the incidence of heteroplasmy in the human population is not fully known. Its occurrence has been used for unique identification (Ivanoc et al., 1996) of certain late stage genetic diseases and there are cases reported of triplasmic humans (Howell et al., 1996).

Figure 5.3: Examples of Various Sequence Artifacts Present in the aDNA Sequences.

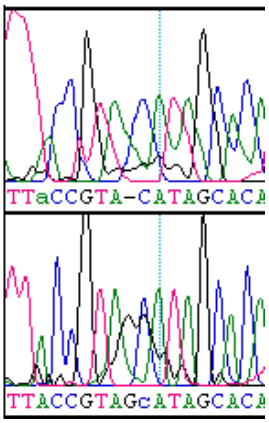
a) VPT2 (G or A) Shown in a Sequence from Sk21 shown in Chromas Pro v1.49.



b) Poor Sequence Segment in Sequence from Sk51

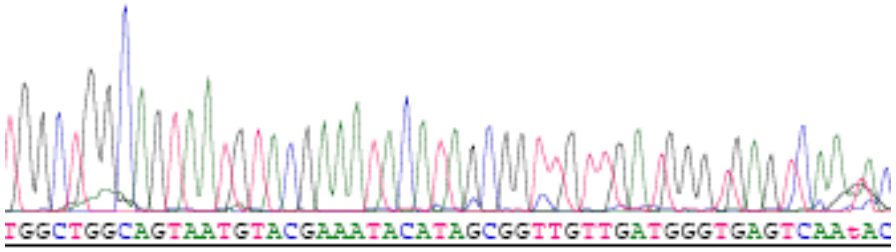


c) VPT1 in a Sequence from Sk21

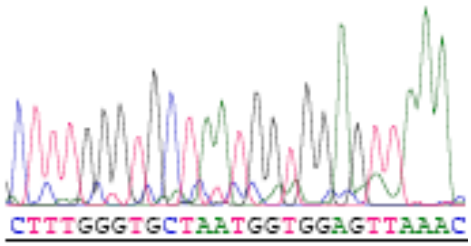


(insertion in lower sequence not present in upper sequence).

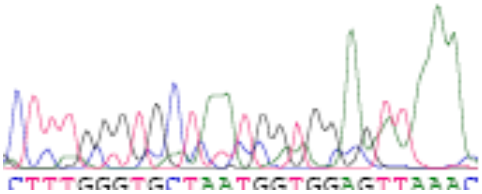
d) Sequential Deterioration of a Sequence Segment from Sk21



e) Background Interference in a Sequence Segment from Sk21.



f) Inconsistent high and low Peak Height in Sequence Segment from Sk21



The 6 traces above are representativ artifacts present in different experimental runs demonstrating commonly occuring aDNA damage expected in ancient tissues.

Microheteroplasmy arises from mutational damage to the mitochondrial DNA which has occurred during the life of the individual. Compared to heteroplasmy, microheteroplasmy is exceedingly common, and increases with age. Each mtDNA has on average 3.3 mutations in coding regions that change the corresponding polypeptide structure. Hence microheteroplasmy must be taken into consideration, particularly when studying individuals of some age (Lin et al., 2002; Parker, et al., 2005; Smigrodzki et al., 2005).

The main factor affecting sequencing results and polymorphic sites is the condition of the template DNA itself. Over time diagenesis comes into effect and causes progressive damage to the mitochondrial genome. As discussed in chapter 1, this can take on many different guises and effects, one of which is the deamination of bases and hence, when read by the DNA polymerase and amplified *in vitro* results in an equal chance of placing either of the corresponding bases into the resultant sequence. Hence, this effect would be most strongly associated with sequencing results as seen in type 2 polymorphic variations.

Polymorphisms within those regions amplified were duplicated by separate amplifications and sequencing. Those positions where anomalous base calling occurred have been given the identity of undetermined pyrimidine, purine or keto depending on the base call variations. Many sequences (over 10) were generated for each sample and due to the number of ambiguous base calls, DNA damage is suspected in these cases. This is to be expected particularly when taking into account the age and state of preservation of the DNA. The results obtained do not match DNA from the principal investigator or according to the specialist laboratory any staff or laboratory user at the sequencing facility. Some DNA damage could have occurred during the extraction and or amplification process.

Table 5.6: Specific HV1 Polymorphisms found in the Towyn-Y-Capel samples.

Sk#	Total SNP#	Definite Mutations	Variable Mutations		Estimated Ht/Hg	
			VPT1	VPT2	Likely	Possible
Sk21	3-5 (5)	C16222T, T16224C, C16270T	C16052Y,	T16311Y	Hg K	Hg U5
Sk33	4-5(6)	C16212G, C16222T, C16224C, C16270T	C16021S	T16311C	Hg K	Hg U5 Hg H (16212)
Sk34	1-5(7)	C16192T	A16252R	G16049A, G16130A, C16221T, C16275T	Hg H21	Hg U5
Sk35	0-2 (3)	None	None	T16311C, C16320T	Hg H	HgK1 (VPT2)
Sk41	1(1)	T16224C	None	None	Hg K*	None
Sk51*	4-5 (9)	T16356C, A16343G, T16342G, G16329A	C16295T	None	Hg U*	U4
Sk52	0 (0)	None	None	None	Hg H*	None
Sk56	1-5(5)	T16224C	None	C16294Y, C16296Y, T16311Y, C16320Y	Hg K1	Hg T* (T2, T4)
Sk57*	1-5(7)	None	C16218T, C16228T, C16250T, A16289C	None	Hg H2	None
Sk60*	0-1(1)	None	T16330K	None	N/D	N/A

List of sequenced skeletons along with the number and identity of SNPs spanning the HV1 region from 16000-16420. In addition to identifying the confirmed mutation from rCRS, also shows possible variable mutations (VPT1 & VPT2) along with the most likely and possible haplogroup estimated from HV1 haplotype. *Partial sequence only (see text)

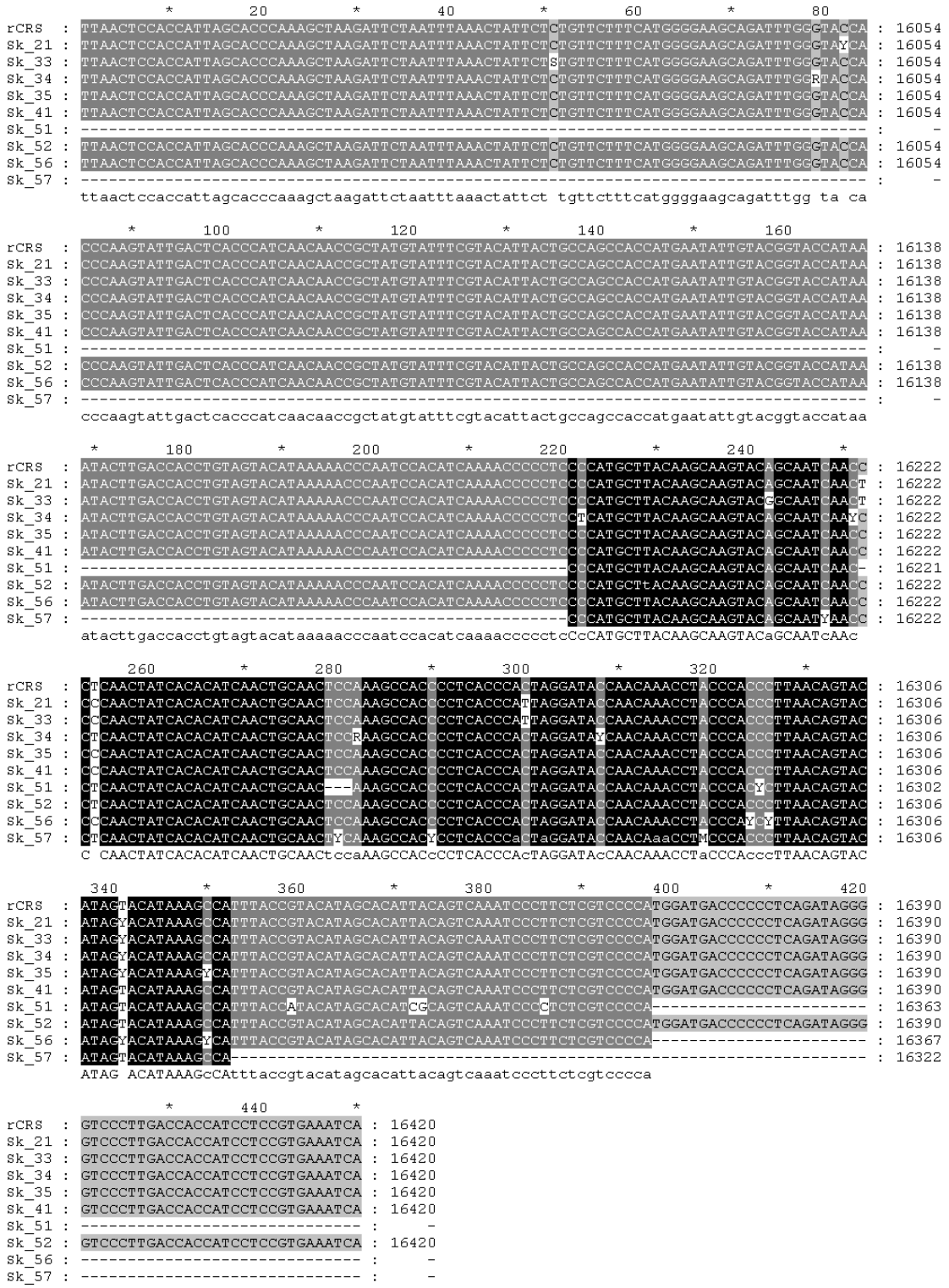


Figure 5.4: Graphical Presentation of Consensus Sequences from Skeletal Samples shown in Genebase Alignment.

Table 5.7: BLASTn Homology Search Matches and Haplogroups.						
Sk#	**Sequence[1]**	**Hits[2]**	**Mutation[3]**	**Score[4]**	**Id[5]**	**Haplotypes Found[6]**
Sk21 (4)	SK 21 Seq A	2	None	826	99%	K2b
A	SK 21 Seq B	13	C16052T	821	99%	K2b, K2a1a H2a2, H*, U5b1d, U5b2
B	SK 21 Seq C	2	T16311C	821	99%	K2b
C	SK 21 Seq D	13	C16052T T16311C	815	99%	K2b, K2a1a H2a2, H*, U5b1d, U5b2
Sk33 (4)	Sk 33 Seq A	2	None	821	99%	K2b
A	Sk 33 Seq B	13	C16021G	815	99%	K2b, K2a1a H2a2, H*, U5b1d, U5b2
B	Sk 33 Seq C	2	T16311C	815	99%	K2b
C	Sk 33 Seq D	13	C16021G T16311C	809	99%	K2b, K2a1a H2a2, H*, U5b1d, U5b2
Sk34 (36)		9	None	828	99%	U5b2, U5a, H3, E4
Sk35 (4)	SK 35 Seq A	90	None	832	100%	K1, K1a, K1a1 K1a4, K1a4a1, K1a4b, K1a6, K1a9, K1b1, K1b2, K1c1,K1c1b, K2a, K2a2, K2b, K3 E13K
A	SK 35 Seq B	2	T16311C	832	100%	K1c1, K1c2
B	SK 35 Seq C	4	C16320T	832	100%	K2a1a
C	SK 35 Seq D	11	T16311C C16320T	826	99%	K1c1, K1c2, K2a1a, H*
Sk41 (1)	SK 41 Seq A	4	None	832	100%	K2a1a
Sk51 (4)	Sk 51 Seq A	1	None	296	97%	K2a1a
A	Sk 51 Seq A	10	None	291	96%	U3.1
B	Sk 51 Seq B	1	C16295T	291	96%	N/A
C	Sk 51 Seq B	1	C16295T	285	96%	U4a2
Sk52 (1)	Sk 52 Seq A	245	None rCRS	832	100%	H*, H1, H1e, H2, H2a2 H3, H4a, H4a1, H7, H7*, H10, H13, H13a, H10, H16, HV5, U5b2
Sk56 (16)	Sk 56 Seq A	4	None	734	100%	U4a2, H1, H2b, K2a1a
A	Sk 56 Seq B	9	C16294T	728	99%	K2a1a
B	Sk 56 Seq C	4	C16296T	728	99%	K1, K1a1, K1a2, K1a4, K1a4a1, K1a4b, K1a6, K1b1, K1b2, K1c1, K1c1b, K2a, K2a2, K3, E13K
C	Sk 56 Seq D	94	T16311C	734	100%	K1c1, K1c2, K2a1a, H*
D	Sk 56 Seq E	11	C16320T	728	99%	K1c1, K1c2, K2a1a, H*
AB	Sk 56 Seq F	22	C16294T C16296T	723	99%	U4a2, H1, H2b, K2a1a, T3
AC	Sk 56 Seq G	96	C16294T T16311C	728	99%	K1, K1a, K1a1, K1a2, K1a4, K1a4a1, K1a4b, K1a6, K1a9, K1b1, K1b2, K1c1, K1c1b, K2a, K2a2a, K2b, K3, E13K
AD	Sk 56 Seq H	17	C16294T, C16320T	723	99%	U4a1, K1, K1c1, K12, K2a1a, H1, H*
BC	Sk 56 Seq I	94	C16296T, T16311C	728	99%	K1, K1a, K1a1, K1a2, K1a4, K1a4a1,K1a4b, K1a6, K1a9, K1b1, K1b2, K1c1, K1c1b, K2a, K2a2a, K2b, K3, E13K

Table 5.7: BLASTn Homology Search Matches and Haplogroups.						
Sk#	**Sequence[1]**	**Hits[2]**	**Mutation[3]**	**Score[4]**	**Id[5]**	**Haplotypes Found[6]**
BD	Sk 56 Seq J	11	C16296T, C16320T	723	99%	K1c1, K2a1a, H*
CD	Sk 56 Seq K	2	T16311C, C16320T	734	100%	K1c1, K1c2
ABC	Sk 56 Seq L	95	C16294T, C16296T, T16311C	723	99%	K1a, K1a1, K1a2, K1a4, K1a4a1,K1b2, K1c1, K1c1b, K2a, K2b, K3
ACD	Sk 56 Seq M	2	C16294T, T16311C, C16320T	728	99%	K1c1, K1c2
ABD	Sk 56 Seq N	30	C16294T, C16296T, C16320T	717	99%	U4a2, K1c1, K1c2, K2a1a, K2b, K3, H*,H1, H5a
BCD	Sk 56 Seq O	2	C16296T, T16311C, C16320T	728	99%	K1c1, K1c2
ABCD	Sk 56 Seq P	4	C16294T, C16296T, T16311C, C16320T	723	99%	K1c1, K1c2
Sk57 (16)	Sk 57 Seq A	1021	None	244	100%	H*, H1, H1a, H1b, H1e, H2, H2a1, H2a2, H3, H4a, H4a1,H6a1, H7, H10, H13a, H16, Hv, HV1, HV5, J1, J1c, J1c3, U2e, U3, U3a, U4, U4a, U4b, U5b
A	Sk 57 Seq B	37	C16294T	244	100%	H* & U304
B	Sk 57 Seq C	1	C16296T	244	100%	N/A
C	Sk 57 Seq D	27	T16311C	244	100%	U304, HV1 & J
D	Sk 57 Seq E	499	C16320T	239	99%	H, H1a,H1, H1e, H2, H2a1, H2a2, H3, H4a1, H6a1, H7, H10, H13a, H16, HV, HV1, HV5, J1c, J1c3,U3, U4a, U4b, U5b2
AB	Sk 57 Seq F	38	C16294T, C16296T	239	99%	H*
AC	Sk 57 Seq G	65	C16294T, T16311C	239	99%	H, H*, HV1, U304 & J
AD	Sk 57 Seq H	134	C16294T, C16320T	239	98%	H*
BC	Sk 57 Seq I	38	C16296T, T16311C	239	99%	H, H1, H3c, H7, HV, U304, J
BD	Sk 57 Seq J	1	C16296T, C16320T	239	99%	N/A
CD	Sk 57 Seq K	27	T16311C, C16320T	239	99%	N/D
ABC	Sk 57 Seq L	66	C16294T, C16296T, T16311C	233	98%	N/D
ACD	Sk 57 Seq M	65	C16294T, T16311C, C16320T	233	98%	N/D
ABD	Sk 57 Seq N	38	C16294T, C16296T, C16320T	233	98%	N/D
BCD	Sk 57 Seq O	30	C16296T, T16311C, C16320T	233	98%	N/D
ABCD	Sk 57 Seq P	66	C16294T, C16296T, T16311C, C16320T	228	97%	N/D

Sk 60 analysis not performed due to the scarcity of complete information.[1] Sequence variation as outlined in §5.2.2, [2]Number of homology hits in the BLASTn NCBI database (www.ncbi.nih.gov/BLAST), [3]Variable Mutation used for each set that varied from the consensus sequence for that sample sequence, [4] BLASTn Point score based on the number of identical base pairs between the enquiry sequence and recovered match sequence [5]Identity in common of the Haplotype sequences found and the sequence investigated, [6]Searches performed using BLASTNn (Basic Local Alignment Search Tool: nucleotide) on all nucleotide sequences submitted or references in the NCBI database; N/A-Not Available; N/D- Not Done.

5.2.2 Consensus Sequence and Polymorphism determination

Consensus sequence results for each sample were carefully constructed, and distinct mutations were assembled alongside the variable mutations in order to fully consider the possibilities from each sequence. The overall consensus sequences are shown in figure 5.4. The entire sequence, including the different variations was compared against the NCBI database using the BLASTn interface in order to determine homology, by comparison with those variations already shown to be present in current human populations. Apart from fragments and partial sequencing of the HV1 region, there were over 4900 entire human mitochondrial genomes available for Hg determination in this database at the time of analysis. Those sequences with the highest degree of homology with sequences in the database are given in table 5.7 and a specific description is given below for each of these samples.

Skeleton 21 BLASTn sequence

Samples from this Sk21 allowed the construction of a full target sequence after the collation of individual sequences. This consensus showed three distinct polymorphisms (as variations from the rCRS) at C16222T, T16224C and C16270T. In addition to these clear polymorphisms, the sequence showed other variable polymorphisms: a VPT 1 change at 16052, possibly corresponding to a C>T and a VPT 2 change at 16311 (an undetermined pyrimidine) possibly corresponding to a T>C variation. BLASTn searches were performed using the Megalign program on the NCBI database and yielded the four possible variations in this sequence;

Sk21 Seq A : C16052, C16222T, T16224C, C16270T, T16311
Sk21 Seq B : C16052T, C16222T, T16224C, C16270T, T16311
Sk21 Seq C : C16052, C16222T, T16224C, C16270T, T16311C
Sk21 Seq D : C16052T, C16222T, T16224C, C16270T, T16311C

BLASTn closest results

Sk21 Seq A: 2 hits with 99% homology K2b

Skeleton33 BLASTn sequence

Successfully sequenced in its entirety, the sequence constructed from Sk33 showed distinct polymorphisms at A16212G, C16222T, T16224C and C16270T. In addition, a VPT 2 change was detected at 16311 ((an undetermined pyrimidine), possibly corresponding to a T>C variation. BLASTn searches performed using the Megalign programme on the NCBI database showed two possible variations of this sequence;

Sk33 Seq A : C16212G, C16222T, T16224C, C16270T, T16311
Sk33 Seq B: C16212G, C16222T, T16224C, C16270T, T16311C

BLASTn closest results

Sk33 Seq A: 2 hits with 99% homology K2b

Skeleton 34 BLASTn sequence

Sk34 yielded a complete sequence, showing a distinct polymorphism at C16192T. In addition, a VPT 1 was detected at 16252 (an undetermined purine), possibly corresponding to a A>G variation and five VPT 2 changes at G16049A, G16130A, C16221T, C16278T and T16311C. BLASTn searching could not be performed due to the degree of variations that these VPTs introduced, due to the degree of data to be ascertained from such large scale searches (3125 different variations).

BLASTn consensus

Sk34 SeqA: 9 hits and 99% homology with U5b2, U5a, H3 and E4

Skeleton 35 BLASTn sequence

Successfully sequenced completely from Sk35, this consensus sequence does not have any definite variations from the rCRS. Possible VPT 2 mutations occur at 16311 (T-C) and 16320 (C-T). BLASTn searches performed through Megalign program on the NCBI database yielded four possible variations of the sequence;

Sk35 Seq A: T16311, C16320
Sk35 Seq B: T16311C, C16320
Sk35 Seq C: T16311, C16320T
Sk35 Seq D: T16311C, C16320T

Closest BLASTn results

Sk35 Seq A : 90 hits and 100% homology with Hg's K1a4, K1b2, K2b, K1b2, K1c1b, K2b, K1c2, K1a2, K2b, K1c1 & K1a

Sk35 Seq B: 2 hits and100% homology with Hg's K1c1 & K1c2
Sk35 Seq C: 4 hits and 100% homology with Hg K2a1a

Skeleton 41 BLASTn sequence

Sequenced completely, this sequence shows a definite polymorphism at T16224C compared to the rCRS. No other differences were observed.

Closest BLASTn results

Sk41 SeqA: 4 hits and 100% homology with K2a1a

Skeleton 51 BLASTn sequence

Samples from Sk51 were incompletely sequenced, with poor quality sequences produced with short fragment length products. Partial sequences between 16252>16367 (Contig 1) and 16167>16262 (Contig 2) were obtained, however. Polymorphic variations included T16356C, A16343G, T16342C, G16329A and the VPT 1 change C16295T. Many of the sequence sections were only amplified twice or show only the single amplification event and so are not consistent or reproducible.

Sk51 Seq A: None
Sk51 Seq A1: None-lower stringency search
Sk51 Seq B: C16295T
Sk51 Seq B1: C16295T-lower stringency search

Closest BLASTn results

Sk51 SeqA: 1 hits and 97% homology with K2a1a

Skeleton 52 BLASTn sequence

Successfully sequenced to yield a full sequence, this shows no difference from rCRS.

Closest BLASTn results

Sk52 SeqA: 245 hits and 100% homology with H*, H1, H1e, H2, H2a2 H3, H4a, H4a1, H7, H7*, H10, H13, H13a, H10, H16, HV5 and U5b2.

Skeleton 56 BLASTn sequence

Almost completely sequenced, and extending from 15970 to 16367of the rCRS, a definite mutation occurs at T16224C. It alsoshows a VPT 2 change at C16294T (a likely T replacement), C16296T, T16311C and C16320T (also highly likely to be a T replacement). Insufficient data was obtained for the entire sequence to be used in a BLASTn search. Partial searches of compiled sequence parts using BLASTn were carried out and yielded the following:

Sk56 Seq A: T16224C
Sk56 Seq B: T16224C, C16294T
Sk56 Seq C: T16224C, C16296T
Sk56 Seq D: T16224C, T16311C
Sk56 Seq E: T16224C, C16320T
Sk56 Seq F: T16224C, C16294T, C16296T
Sk56 Seq G: T16224C, C16294T, T16311C
Sk56 Seq H: T16224C, C16294T, C16320T
Sk56 Seq I: T16224C, C16296T, T16311C
Sk56 Seq J: T16224C, C16296T, C16320T
Sk56 Seq K: T16224C, T16311C, C16320T
Sk56 Seq L: T16224C, C16294T, C16296T, T16311C
Sk56 Seq M: T16224C, C16294T, T16311C, C16320T
Sk56 Seq N: T16224C, C16294T, C16296T, C16320T
Sk56 Seq O: T16224C, C16296T, T16311C, C16320T
Sk56 Seq P: T16224C, C16294T, C16296T, T16311C, C16320T

Closest BLASTn results

Sk56 SeqA: 4 hits and 100% homology U4a2, K2a1a, K2b & H1
Sk56 SeqC: 94 hits and 100% homology with K1c1, K1c2, K2a1a & H*
Sk56 SeqK: 2 hits and 100% homology with K1c1 & K1c2.

Skeleton 57 BLASTn sequence

This skeleton yielded only partial sequences, extending from 16191 to 16322 of the rCRS. These show VPT 1 mutations at C16218T, C16228T, C16250T and A16289C. Insufficient data was obtained for an entire sequence BLASTn search, but partial searches of compiled sequence parts were carried out using BLASTn.

Sk57 Seq A: None
Sk57 Seq B: C16218T
Sk57 Seq C: C16228T
Sk57 Seq D: C16250T
Sk57 Seq E: A16289C
Sk57 Seq F: C16218T, C16228T
Sk57 Seq G: C16218T, C16250T
Sk57 Seq H: C16218T, A16289C
Sk57 Seq I: C16228T, C16250T
Sk57 Seq J: C16228T, A16289C
Sk57 Seq K: C16250T, A16289C
Sk57 Seq L: C16218T, C16228T, C16250T
Sk57 Seq M: C16218T, C16250T, A16289C
Sk57 Seq N: C16218T, C16228T, A16289C
Sk57 Seq O: C16228T, C16250T, A16289C
Sk57 Seq P: C16218T, C16228T, C16250T, A16289C

Closest BLASTn results

Sk57 Seq A: 1021 hits and 100% homology with H*, H1, H1a, H1b, H1e, H2, H2a1, H2a2, H3, H4a, H4a1,H6a1, H7, H10, H13a, H16, Hv, HV1, HV5, J1, J1c, J1c3, U2e, U3, U3a, U4, U4a, U4b, & U5b.
Sk57 Seq B: 37 hits and 100% homology with H* & U304
Sk57 Seq C: 1 hit and 100% homology with GenBank: U47206.1 (Ht information not available), geographical origin Polynesian (Sykes et al., 1995)
Sk57 Seq D: 27 hits and 100% homology with U304, H, HV1 and J.

Skeleton 60 BLASTn sequence

This skeleton yielded amplicons for sequencing of extremely poor quality, and only data of short sequence length was produced. No verifiable genetic data could be recovered from sequences, although some recovered fragments permitted partial searches of compiled sequence parts which were carried out using BLASTn. Some homology was discerned with Hts with J2a, H (100%), H1a1 and J1c.

5.2.3 Sequence Comparison against a Population Database

Sample mutations were compared against the larger though non-geographical or subclade specific genographic database from the National Geographic results of the Genographic Project. This is the world's largest and most comprehensive public mtDNA determination project to date, and contains 50033 HV1 sequences and corresponding SNP's used for Hg determination at the time of use (Behar et al., 2007). A summary of mutations and variable mutation found with associations to other mutations and Hgs is given in table 5.8. A specific description of the individual samples is also given below.

Skeleton 21 Sequence

16222T, 16224C, 16270T from this sequence are consistent with the HV1 Ht polymorphism for human mtDNA Hg K. Even when combined with the possible change 16311Y, correspond to 32/50033 samples in the genographic project, always associated with the 16311C transition and the unsequenced 16519C.

Skeleton 33 Sequence

The 16212G mutation in this sequence is very specific and related only to Hg H, identified in 25 out of 50033 individuals from the genographic project database. However, the other mutation at 16224 is not seen in this population subset, the possible 16311 variation. This may be a unique genotype or a spurious result. The transitions at 16222T, 16224C, 16270T concord with HV1 Ht for human mtDNA Hg K.

Skeleton 33 Sequence

The unusual mutation central to the Sk33 sequence is the 16212G transition, which has been associated with a few distinct populations, mainly isolated aboriginal cultures, such as the Thules in Greenland (Saillard et al., 2000), Slovenians (Pajnic et al., 2004) asians in Japan (Uchiyama et al., 2007) and Anatolians in Turkey (Mergen et al., 2004). The remaining variations and possible heteroplasmy corresponds to HV1 Ht of Hg K (16224 and 16311) and with the U5, a Hg K predecessor at the 16270 mutation. Internet research shows a U5b variant with the 16222 and the 16270 SNPs[5].

[5] http://old.worldfamilies.net/mtdna/u5/results.html

Table 5.8: List of Polymorphisms Found in Towyn-Y-Capel Sequences.

rCRS Position	Sk#	rCRS > SNP	SNP Type	Associated SNPs	HV1Haplotypes/ Haplogroups	References
16021	Sk33	C>Y(S)	VPT1	N/I	N/A	None
16049	Sk34	G>R	VPT2	N/I	N/A	None
16052	Sk21	C>Y	VPT1	16126C, 16292T 16294T	Hg T3	Appendix**
16130	Sk34	C>M	VPT2	None	Cold Spot*	Malyarchuk, 2007
16182	Sk34	C>T	SNP	16184A, 16189C, 16270T, 16311C, 16336A	Hg U5b	Appendix**
16192	Sk34	C>T	SNP	None	Hg H21	Appendix
				16270	Hg U5	
16212	Sk33	A>G	SNP	Single mutation	Hg H*	Appendix**
16218	Sk57	C>Y	VPT1	Non specific	N/ A	Appendix**
16221	34	C>Y	VPT2	Single Mutation	Hg's H* & HV*	Appendix**
				Others Non Specific	Hg's U5a1a, R*, M*, I3*, N9a & W*	
16222	Sk21, Sk33	C>T	SNP	16244, 16270, 16311	Hg K*	Appendix**
				16311	Hg H*	
				Single Mutation	Hg H* & Hg V*	
16224	Sk21,Sk33, Sk35, Sk41, Sk56	T>C	SNP	16311 16320*	Hg K*	Appendix**
16225	Sk51	C>T	N/A	N/I	Cold Spot*	Malyarchuk, 2007
16228	Sk51	C>T	N/A	N/I	Cold Spot*	Malyarchuk, 2007
	Sk57	C>Y	VPT1			
16245	Sk51	C>T	N/A			Appendix**
16250	Sk57	C>Y	VPT1	16463G	Hg H*	Appendix**
16252	Sk34	A>R	N/A	N/I	Cold Spot*	Malyarchuk, 2007
16259	Sk57	C>Y	N/A	N/I	N/A	None
16260	Sk57	C>Y	N/A	N/I	N/A	None
16270	Sk21,Sk33	C>T	SNP	16192*	Hg U5	Appendix**
16278	Sk34	C>Y	VPT2	16311	Hg H11	Appendix**
				16189	Hg X	
16289	Sk57	A>M	VPT1	None	Hg H*	Appendix**
16294	Sk56	C>Y	VPT2	None	Hg T*	Appendix**
				16153, 16296 & 16304	Hg T2	
16295	Sk51	C>Y	VPT1	None	Hg H*	Appendix**
				16311T	Hg HV	
				16356T	Hg U4	
				Others	Hg's V & N	
16296	Sk56	C>Y	VPT2	16153 16294 16304	Hg T2	Appendix**
16311	Sk21,Sk33, Sk34, Sk35, Sk56	T>Y (C)	VPT2	16224, 16320*	Hg K	Appendix**
				16278	Hg H11	
16320	Sk35, Sk56	C>Y	VPT2	16224, 16311	Hg K1	Appendix**
16329	Sk51	G>R (A)	SNP	N/I	Cold Spot*	Malyarchuk, 2007
16330	Sk60	T>K	VPT1	N/I	Cold Spot*	Malyarchuk, 2007
16342	Sk51	T>C	SNP	None	Hg U* & H*	Appendix**
				16343G 16353C 16390A	Hg U4	
16343	Sk51	A>R(G)	SNP	None	Hg U3	Appendix**
16354	Sk57	C>T	N/A	N/A	N/A	None
16356	Sk51	T>C	SNP	None	Hg U4	Appendix**

Table lists all the polymorphisms found among the Towyn-Y-Capel sequences including type (SNP/VPT/VPT2) and with which haplogroup (or groups, if any) it is associated to along with references.*Malyarchuk, B. (2007) Cold spots of human mitochondrial DNA hypervariable segment 1. Molecular Biology. 42(3); 399-402; **Appendix 5.2; N/A : Not Available; N/I : No Information.

This is consistent except for the 16222 SNP which, according to the genographic project, only occurs in 25 individuals of the total of 50033 DNA samples match and none of them match with the remaining transitions in the same sample (0.056% of the genographic project). Therefore, according to the Hg variation in genographic project this is an uncommon variant of the H Hg. 16311 SNP is a defining mutation, either for the H11 subclade of Hg H, or for Hg K, along with the other mutation 16224. The transition 16222 is associated with a broad section of Hgs from H and K to U, V, and W. Although the combination of 16222T, 16224C, 16270T, 16311C shows a Hg K (28/50033). This could represent either a miscoding lesion, contamination or an unusual Hg K Ht. Hg K has only two subclades, neither of whom have their defining mutations in the Sk33 sample sequence.

Skeleton 34 Sequence

16192T, which was only found to be present in Sk34, was linked to a large number of Hts: U5, U5a, U5a1a, U*, HV*, W, R*, F, H, D, H, V, U3, U4, L3e2, K, U1, L2c, M*and N*. The only other is a possible variation at the SNP 16311C, which narrows the field down from hundreds to 59, mainly U*, possibly HV* and/or H that is mainly associated with 16519C mutation (not done) although some U* also have that particular mutation.

Skeleton 35 Sequence

This corresponds to the rCRS Hg H, with an additional mutation at T16224C. The sequence is consistent with K1 Ht in the HV1 region in the genographic project, where 184/50033 Hg K1 individuals have the same sequence profile (0.36% of the total population). If the possible mutations at T16311C and C16320T are taken into consideration, only 2 individuals match the entire profile: one Hg K individual and one Hg H.

Skeleton 41 Sequence

This corresponds to the rCRS, with a definite mutation at T16224C that is present in many samples. However, without any matches for SNP 16350, mostly Ht K (with 16519C) corresponding to 8/50033 individuals, 5 individuals with Hg H, 3 individuals with Hg H and the 16519C SNP in 1 Hg U* individual with 16519C.

Skeleton 51 Sequence

This sequence presented a more complex situation in terms of Hg matching in that there was a large number of sequence variations, particularly the mutations at 16222T, 16225T, 16228T, 16245T, 16342C, 16356C and the possible mutations 16329R and 16343R.

For 16222T, a single individual had a “stand alone” SNP at this position, but was not associated with the SNPs at 16225T, 16228T, 16245T, 16342C or any of the others but was associated with other SNPs in Hgs H, W, K & V. 16225T and 16228T were not found as a variation in the genographic project. 16245T occurs but is very rare (only 3/50033 sequences). A further two are associated with Hg H and 1 with Hg.

The 16342C mutation corresponds to the three Ht H, U4 and U*.U4 differs in also being consistent with a 16343G modification and the 16356C mutation, which supports both one of the definite mutations and a possible SNP. U4 determination therefore for the sequence looks the strongest match. 16356C is most commonly associated with Hg U4, although it does occur within a small population of Hg H (a rare group or early progenitor on a different branch). 16329R SNP does not appear (nor any transitions there) and16343G represents mainly U3 or U4 Hgs, occurring only in 1 Hg H and 1 Hg U* as a variation.

Skeleton 52 Sequence

This sequence corresponds exactly with the HV1 Ht for the rCRS Hg H.

Skeleton 56 Sequence

This corresponds to the rCRS with an additional mutation at T16224C and four possible mutations at 16294Y, 16296Y, 16311Y, 16320Y.

Skeleton 57 Sequence

A possible change in base early for the troublesome Sk57, common in a large number of Hgs, from H, U*, V, U5, to A and L3*, K, D, C, M* this sequence is not very specific. As a heteroplasmic samples though it's specific for U5a1a, however the confirmatory transitions at 16256T and 16270T are missing. Next possible 16228Y does not correspond to any sample either, 16250Y neither, nor 16259Y, 16260Y however matches with a 16260T for a Hg H sample (only the single individual with another variation at 16328a (not shown in the analysis) and a 16519Y (not sequenced).

Skelton 60 Sequence

The 16330K mutation detected in some trace sequences does not correspond to any SNP detected in the genographic project.

5.2.4 Summary of the Compiled Information from the Towyn-Y-Capel Sequences

The results of the sequence and point mutation analysis are given in table 5.6, with the nature of the mutations and predominately associated mutations with the determined HV1 Ht and putative Hg.

Given the degraded nature of the templates, the multiple polymorphisms and the inherent susceptibility of aDNA samples to contaminant sequences, a degree of caution should be associated with the analysis, interpretation and conclusions drawn from these sequences. In many cases, it appears that the variable mutation (either VPT1 or VPT2) may result from single base diagenetic degradations, and therefore may not be interpreted other than by comparison with other sequences and by deductive reasoning. Mutations such as T16330K (Sk60), for example, are not found in the databases searched, and therefore represents a "cold spot", not normally susceptible to mutation and likely therefore to be an artefact of the nature of the aDNA template, rather than a defining mutation of the associated individual (Malyarchuk, 2007).

Other cases of VPT that result from artefact formation may not be so easily distinguished, and this must also be considered, particularly when there is an arbitrary mutation not normally associated with the predominant mutations. Another consideration is that of possible contamination, and multiple sequences may be detected, such as in the samples from Sk56, where the VPTs correspond to the Hg T, but the main mutation is associated with Ht K. This leads to the possible conclusion that more than one genetic template may be present, with at least one from a contaminant source.

An interesting aspect regarding the VPT detected is that they frequently occur at positions that correspond to known Ht SNPs. These may indeed be mutational hotspots, that either due to their chemical, structural or conformational nature and/or immediate environment on the DNA strand, are more susceptible to mutation, whether in-vivo or in ancient remains (Stoneking, 2000).

On the important aspect of possible contamination, the sequences from Towyn-Y-Capel are suitably variant and the overall condition of the sequences support aDNA origin. They also appear to be part of a European population, in that Hts K, U and H all have established European lineages and form part of the normal distribution of both ancient and modern populations. Hts K and U, in particular, are found in a large proportion of the sequences

retrieved and it is of particular note since they correspond to the earliest populations estimated to have been present in the northernmost and westernmost parts of Europe.

There is a concern however, particularly with regards to samples from Sk21 and Sk33, is the predominance of Ht K corresponds to the key investigator's HV1 Ht. These concerns are somewhat relieved due to other plausible mutations within the sequences that do not relate to the investigator's sequence. However, this remains a possibility, particularly in light of the earlier comprehensively negative results regarding these samples. By necessity therefore, some doubt must also be given to the results relating to the Ht H, particularly sequence from Sk52, which shows complete similarity with the rCRS H2a2 sequence, often used as a control and reference sequence, and is a significant part of the modern European population (Figure 6.1).

Possibly one of the most important sequences of the investigation with regards to the research and the conformation of the aDNA sequences as genuine. All samples were handled and manipulated by this single researcher from removal from the excavation to the amplification of the aDNA extracts. Important deviations from this are with regards to the paleoDNA research, where the chain of possession no longer remains with the key investigator. As previously described there is some similarity in some of the sequences (Table 5.6) and the positive control (rCRS Ht H) or the investigators DNA (Ht K) although no sequential similarity is found between the m all, so there is no clear evidence of contamination from either source.

5.2.5 Intra-population Distribution and Phylogenetic Variation

From the assembled consensus sequences the degree of variation within the samples themselves and from the rCRS can be represented graphically as a simple branched phylogenetic tree (Figure 5.5) compared to the rCRS or the more complex Network assemblage of figure 5.6 within the Towyn-Y-Capel sequences only. Sequences were aligned manually. Phylogenetic relationships were estimated using median-joining networks (Bandelt et al., 1999) as implemented in Network 2.0d[6] and refined by hand. The same topology was obtained using the neighbour-joining method.

[6]http://www.fluxus-engineering.com

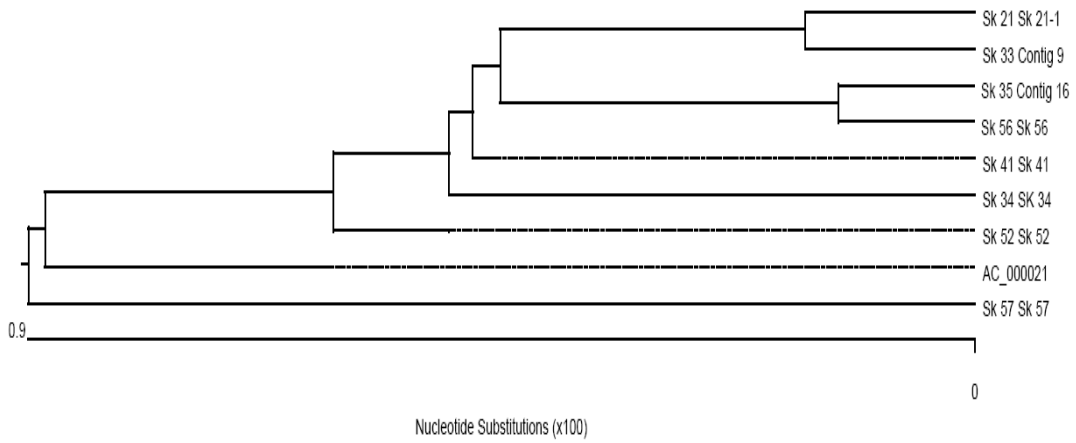


Figure 5.5: Simple Conservative Inter Population Phylogenetic Tree.
Construction using confirmed mutations to illustrate inter sequence variation between samples and the revised Cambridge Reference Sequence (AC 000021)(Clustalw).

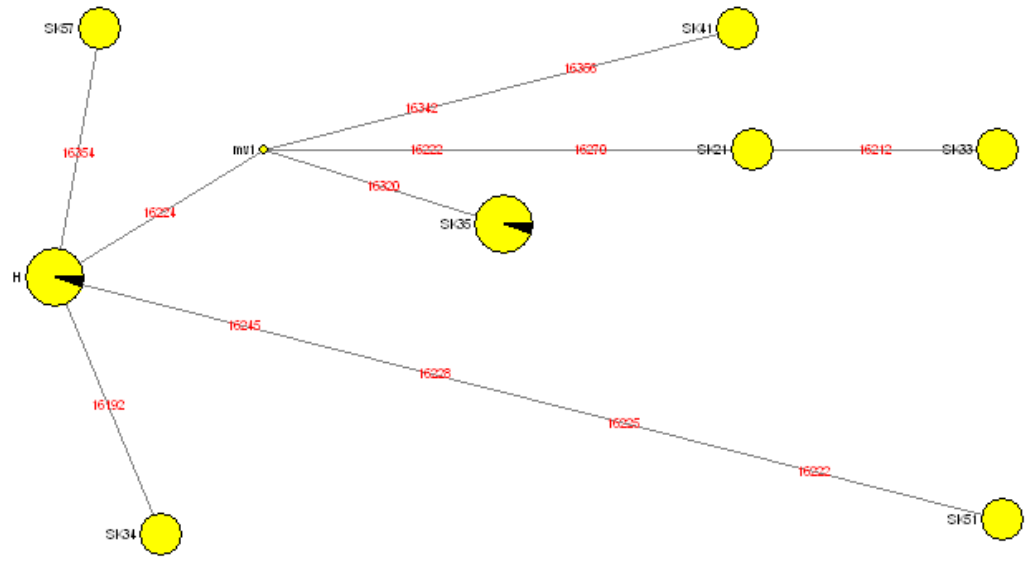


Figure 5.6: Conservative Phylogenetic Tree Reconstruction.
Population tree was put together using confirmed mutations (Table 5.1).Tree constructed using proprietary software network 2.0 and base pair difference (excluding ambiguous base calls based on non-verifiable VPTs.) Sk60 excluded due to partial fragmented sequence.

5.2.6 Sequencing Conclusion

It appears that the higher amplification rates possible at specialised ancient DNA laboratories, have led to the amplification of some aDNA sequences from human mitochondria. These have yielded the successful amplification and sequencing of nine of the ten skeletal samples in the mtDNA hypervariable 1 (HV1) region with varying levels of success.

The sequences obtained bear all the characteristics expected from highly degraded ancient DNA, such as low sequence peak heights, high background and numerous miscoding lesions. These sequence results were qualitatively analysed, and compared against established modern populations and publications to reconstruct the original sequence.

The sequences within the population are varied, and comprise the predominate Ht K, U and H, which is consistent with the presence of older north Atlantic population in Hgs K and U, and the more recent European population, through the currently predominate Hg H.

The verifiability of the sequences is, however, like most ancient investigations, overshadowed by the need for some degree of caution, particularly given the lack of success using a variety of other amplification profiles at earlier stages in this study, and given also the possibility of contamination.

5.3 Overall Genetic Conclusions

In marked contrast to results achieved in chapter 4, the independent assays described in this chapter were overwhelmingly successful. Nine out of the ten sequences, were recovered successfully and entire HV1 sequences were recovered from six out of the ten sequences. Apart from variations in the lysis, extraction and amplification protocol, the main factor considered to be responsible for this unexpected success was the PCR profile which was in excess of 50 cycles. This level of over amplification was not attempted at the UCLan laboratories as the conditions required to successfully amplify at this level are dust and DNA free laboratories, where the background level of genetic contamination is significantly reduced. This was not available at the UCLan, and those that were available in the earlier part of the research were not available later due to reallocation of research facilities. Of course the marked success rate is also a reflection of the selection criteria used for these samples from amongst the others (see chapters 3 & 4).

Analysis of the sequences successfully recovered showed all the trademarks of aDNA including miscoding lesions and low peak height and significant background, which makes ascertaining relevant variation information from the samples difficult. In light of the small sample size, a qualitative and logical analysis of all variations due to “possible” base variations at the presence of miscoding lesions, including looking at the different sequences overall and the point mutations themselves and where and with whom the mutations are associated. End results showed the predominant HV1 Hts and associated (but unsubstantiated) Hgs. The population shows Eurocentric origin, with U, K and H Hgs like HV1 Hts, with a population distribution that is higher in the U/K Hg (as K is a subclade of the Hg U) than in the modern populations, however with such a small sample size this may be unrepresentative. The results, however, must be treated with scepticism, the fact that they were not confirmed by the UCLan amplifications, and the inherent similarity in trace contamination sequence profiles with aDNA sequence profiles belies possible contamination.

CHAPTER 6

GENERAL DISCUSSION

6 GENERAL DISCUSSION

The multidisciplinary nature of this research study was focussed mainly on the taphonomy (§2), molecular chemistry (§3) and molecular biology (§4 & §5) of the Towyn-Y-Capel remains. In order to study the Towyn-Y-Capel remains as completely as possible the project also embraced aspects from the fields of dentistry, histology, genetics, organic, inorganic and geo-chemistry. The use of multiple lines of enquiry was imperative given the complexity, variability and uncertainty associated with the retrieval of genetic material that was the primary aim of this research. From the very first genetic experiments, the necessity of a considered multidisciplinary approach was evident, in light of the many issues associated with ancient DNA research. This approach maximised the information retrieved from the archaeological remains.

The research itself was divided into two different sections: screening experiments and information gathering experiments. Screening experiments provided valuable information regarding the state of the sample itself, especially concerning the structure, condition and preservation of the individual and collective skeletal elements with some comparison to other populations and modern controls. Information experiments involved the acquisition or recovery of the data from the skeletal elements to gain information about the particular individuals and the whole population.

Both sets of experiments are mutually important in the study of fossilised remains. Screening techniques, for instance, often include steps such as photography and recording which are essential in dealing with unique and irreplaceable research material, while subsequent information gathering experiments, which are usually desctructive in nature, such as stable isotope analysis, trace element profiling and DNA sequencing can provide valuable information regarding the diet, origins and migrations of the individuals and, by extrapolation, the whole population.

It is unfortunately the case that most scientific studies of ancient remains concentrate on only one feature and not the other, possibly because of the diverse expertise and experience required.

Nonetheless, one of the key precepts in this research is the importance of undertaking a comprehensive and dynamic approach, including the use of screening and information experiments, for the study of ancient tissues, particularly when considering ancient DNA and

the use of unique samples. For the sake of continuity with the previous chapters of this thesis, this chapter discusses the main points relating to the previous chapters in sequential order, before bringing together the conclusions from the different lines of enquiry to give a full picture of the research undertaken.

6.1 The Difficult Nature of aDNA Recovery

Ancient DNA (aDNA) is a particularly difficult molecule to study, due to its low concentration and poor quality in ancient biological samples. In skeletal remains, the degraded substrate is bound to the bone or tooth matrix, often in the presence of numerous poorly understood PCR inhibitors, and the additional complication of contamination by other DNA molecules. In addition, aDNA is almost exclusively unique, constituting rare fragments of a genetic history that can provide insight into our past. Due to this rarity and the potential for contamination by modern sources of DNA, extreme caution is necessary in the sampling and analysis of aDNA material.

This research provided a unique opportunity to study an isolated medieval Welsh population. This study involved direct sampling from the excavation site in order to accumulate the large skeletal collection to work with. This was a rare opportunity in biomolecular archaeology, as most samples are museum archives that have been previously handled by many archaeologists and have little to no association with the original context. This excavation provided an opportunity to avoid one of the most common sources of aDNA contamination, namely that of sample handling. With this in mind, a considered approach was taken to sampling and in ascertaining the condition and preservation of the material itself, to permit subsequent consideration of the suitability of samples for aDNA extraction.

6.2 Anthropology and Taphonomy

Taphonomy was once the mainstay in the study of archaeological remains up to the late eighties and early ninties (Haglund & Sorg, 1997) but since then, it has been complimented by the increasing use of biomolecular techniques, such as genetic profiling, trace It has retained its usefulness for analysing remains for determination of physical characteristics, gender, disease, lifestyle and, in some cases, cause of death, and has also been recently used as a screening system, particularly for selecting samples for genetic analysis.

6.2.1 Sampling, Cataloguing, Maintenance & Selection

With the increasing availability and sensitivity of biomolecular techniques, far greater information can now be ascertained from archaeological remains than ever before. Unfortunately, these techniques normally require the destructive sampling of irreplaceable remains. Previously, this issue has been resolved by the use of trace amounts of non-essential or osteologically unimportant archaeological samples. However, these samples, as shown in §2.2.1; §3.4.1, do not necessarily provide the best samples for analysis. For example, teeth have been shown to be optimal for aDNA extraction and essential for trace element analysis and isotope ratio determination, due to the greater post mortem diagentic modification of bone (Ricaut et al., 2005; Hoppe et al., 2003; Budd et al., 2000; Kohn et al., 1999), although they can also provide a great deal of anthropological information, such as age, health and diet. In order to address this issue, it is necessary to keep a comprehensive record of the samples and record as much information as possible for future analysis. All human teeth and bone samples were therefore recorded by high quality photography (§2.2.1; Appendix #2.2), which should be a standard amongst all laboratories, to obtain a detailed record to support potential further research.

6.2.2 Site Sampling

Sampling involved the asceptic removal of the bone and tooth samples during the excavation itself, before their potential exposure to modern human DNA, which is often the prime source of contamination, and which unlike laboratory contamination, cannot be easily detected. This avoids the problems of museum collections, for example, where multiple anthropologists have handled the calcified tissues leading to, contamination and rendering the sample unusable as a source of aDNA. Site sampling is now a standard technique in Forensic Archaeology, and it was the experience of this investigator that awareness and foreknowledge of contamination issues amongst the archaeologists assisted greatly with the site sampling.This mirrors the chain of custody used for forensic samples in that, from the sample collection to processing, this single researcher was solely responsible for extraction, storage and processing of the samples to minimise contamination.

6.2.3 Sample Selection

The most important factors pertaining to the selection of samples from the site were the use of appropriate tools and prior arrangement with the on-site Anthropologists and Archaeologists carrying out the excavation, and the integrity and condition of the skeletal material.

The preferred tissues were teeth, and of these molars, preferably 2nd or 3rd mandibular (lower) molars and ideally two molars from each skeleton, in order to independently duplicate the results. An important point to consider with tooth sampling is the need for the use of the correct tools. Dental forceps are best used for removing teeth, along with the appropriate technique best learnt in collaboration with a local dentist or dental school, and, where possible the extraction of lower molars in preference to upper molars, due to differences in the root structure.

The researcher must be familiar with photographic techniques, tooth anatomy and the appropriate mounting and cleaning techniques, all without contaminating the samples. Thus, a same sample may be photographed, and used for non-destructive analysis before use in DNA, stable isotope and amino acid analysis.

6.2.4 Loose finds

An important benefit of being present at the site of the excavation was the selection of loose finds for initial research. Normally found at archaeological digs are a selection of non-associated remains of bones and teeth that cannot be matched to a particular assemblage and these are commonly called “loose finds”. These are normally remains of animals that have burrowed into the archaeological area itself or may be present for a number of other reasons at the site, perhaps related to the archaeological or chronological stratigraphy of the site. In burial mounds such as Towyn-Y-Capel, that have undergone disruptions, “loose finds” also include scattered human remains from disturbed graves that cannot be identified. These “loose finds” are useful for a number of reasons. They are excellent samples for histological analysis and as site controls for stable isotope and amino acid racemization, particularly when they are teeth. They can give excellent information regarding local diagenetic effects, isotope ratios, and in can be used for genetic spiking experiments for optimising genetic protocols. It is also useful to take at least one soil control of the burial area for later analysis for further insight into the biological, chemical and isotopic makeup of the surrounding soil.

6.2.5 Sample storage

Sample storage must be carefully thought out, as ancient tissues need to be ventilated (kept dry to prevent mildew forming) and sealed against further contamination particularly in transit. This was accomplished by using sterilised brown paper bags held in perforated plastic bags to prevent moisture accumulation. The dry sandy soil of the site did not provide much of an issue in this respect, and also teeth are more resilient than bones to this type of damage.

Samples were kept in the original state under ambient temperature, until ready for processing, and when processed, samples were manipulated within days of handling within the laboratory. Sections were separated for the individual chemical analysis (§2.2.1; §3.4.2; §4.3) and the remainder pulverised and kept as prealiquoted bone powder at -20^{o}C until extraction.

6.2.6 Gross & Histological Preservation Indices

Once collected from the site and taken to the laboratory, samples were evaluated on the basis of their preservation. Although not strictly related to the subsequent successful extraction of biomolecular material, it is usually the case that skeletal elements with poor gross or histological structure are unlikely to provide good molecular results, particularly concerning DNA. Unfortunately, the opposite is not necessarily true, although generally, well-preserved samples provide the best opportunity for successful analysis. A benefit of the recoding protocols was the independent assessment of the gross preservation index of the samples through multiple independent scoring achieved without the direct handling of the samples themselves. From this a more reproducible score can be achieved from a qualitative technique. Unfortunately, due to the lack of success in reproducibly extracting gentic material, the potential for genetic analysis cannot be closely matched to the preservation condition of these samples. Research by Haynes and Coll (2002) supports the notion that poor preservation and poor aDNA condition are linked, although good preservation does not necessarily indicate good molecular condition.

With regards to the histological condition of the teeth, the primary work done was performed on loose finds, due to the large amount of material required for the destructive technique, difficulty in the crosssectioning of patella, due to the spongiform nature of the bone, and teeth, due to the hardness, and lack of access to specialised histological equipment such as the cryostat. The simplified grinding technique used for the animal samples, coupled with a basic soil analysis provided a basic overview of the histological condition of the bones (§2.3.6) showed a generally high level of preservation, with all the structural and cellular components intact. The bone preservation did, however, show evidence of infiltration, mainly seen in the colour difference between the bone surface and deeper cortical sections and also some fissures, cracks and focal tunnelling.

6.2.7 Biochemical Condition of Calcified Tissues

A novel non-destructive technique used was the autofluorescent analysis of teeth, QLF, as described in §2.2.3. This technique although based on a principle that despite being poorly characterised, has seen increasing popularity in recent dental research for detecting incipient dental decay (Stookey, 2004; McConnell et al., 2007), although this has not been used previously for archaeological or forensic purposes. Autofluoresce is associated with the calcium content in teeth, and as such was used to determine the integrity of the inorganic structure of the teeth from the Towyn-Y-Capel site (§2.3.5). The benefit of this technique is its non-destructive nature, and, as with the ancient sample photography, the value as a permament record of the tooth prior to destructive analysis. The results, concentrating on the analysis of the occlusal surface, showed some deterioration in the fluorescence intensity in most Towyn-Y-Capel samples. This result was relative compared to modern control teeth and indicated that apart from organic degradation the inorganic faction samples also showed degradation, or possibly modification. Research undertaken with *bovine* incisor models comparing both X-ray transmittance and the release of calcium into solution, showed that fluorescence only matches the initial drop in calcium in the tooth matrix, and that fluorescence remains in the teeth. The remaining fluorescence is of a lower intensity, although it persists even when tooth was fully demineralised. The technique looks promising, as most of the dental research, although associated software applications work on the principle of comparative localised demineralisation (in healthy undamaged (control) quadrant on the same tooth) which needs to be addressed. The main problem is the unknown nature of the fluorescent molecule itself, which although clearly associated with the mineral phase of the tooth structure is also independent of the calcium structure. The structure, distribution and associations with the hydroxyapatite need to be better understood before information gained from this technique can be fully evaluated.

6.3 Extraction & Amplification of Ancient DNA

The main focus of this research was the attempt to retrieve genetic information from the bones and teeth of the skeletal individuals of the Towyn-Y-Capel population. Unfortunately, the sandy soil conditions, coupled with the age of burial and predominant conditions, coupled with the scarce and rapidly degraded nature of the endogenous DNA, compared to the amino acids present in collagen or the trace elements in the hydroxapatite structure, also mean it was the most challenging. In its shadow a great deal of information has been elucidated, particularly with regards to appropriate selection of the calcified tissue as a source of DNA.

Patellae, for example, are not a good source of endogenous DNA (or practically any biomolecular application and should be clearly avoided), and some novel techniques such as the use of RAPD-PCR.

6.3.1 Preliminary Genetic Analysis and the Control of Contamination

Following the initial sampling period, preliminary analysis of the laboratory, procedures, and consumables was undertaken. For the assessment of contamination of consumables and non-human site samples, HV1 specific human mitochondrial primers were used to amplify a 412 base pair (bp) product. This is considered to be the uppermost extent of the fragment size commonly found in aDNA research. Initial samples were extracted using the popular forensic procedure called chelex extraction. This extraction technique is renowned for its simplicity and reliability. The initial results were promising; no contamination was detected and two human tooth samples showed positive results (other human tooth and two human bone samples gave negative results). This is consistent with those views expressed by authorities in the field, which hold that teeth provide a fbetter source of aDNA than bone. Tooth enamel and dentine is far more resistant to the long term diagenetic effects of the environment and microbial attack. Additionally, the PCR inhibitors commonly found in bone do not seem to be as prevalent in tooth samples, probably again due to the protective nature of dentin. The consensus from initial experiments however, looked favourable for successful amplification of mtDNA sequences.

6.3.2 Randomly Amplified Polymorphic DNA Analysis

At this initial stage of the investigation, methods were selected according to the literature and availability of material, but had not yet been fully optimised. In addition, only limited information is available on the genomic integrity and the nature of some of the site samples. Hence, it was decided to optimise the procedures for DNA extraction and amplification. Procedures were optimised by using ancient material (non-skeletal loose bone) from the site to avoid consuming any of the more important catalogued skeletal material. This material came from a variety of biological sources, from human to bird bones. RAPD analysis of these samples would therefore involved a wide variety of unknown genomic templates, and unique banding patterns (or fingerprints) for each species was anticipated. Initial results were not ideal, as random banding patterns of less than two bands were obtained, which did not therefore permit identification at the species level.

To improve the poor amplification results, DNA was extracted using both the QIAmp Mini kit and the Geneclean aDNA kit instead of chelex. The aDNA kit produced the best results and was used for all following extractions. This success was probably due to stringent purification steps in the Geneclean kit that may have removed more PCR inhibiting materials from the aDNA complex. Subsequent RAPD reactions were very successful, producing up to 15 identifiable bands depending on the primers used. However, aDNA banding patterns were mainly very weak and barely detectable, or else gave DNA bands of large molecular weight (around 1000 bp). The main problem found when analysing the RAPD fingerprints for each sample was the lack of commonalities in the banding patterns. Indeed, no observable consistencies occurred, and no samples adequately matched the 6 standards; human, pig, sheep, dog, deer, and rat. Although this is not an impossible scenario, it is highly unlikely that no samples, human or otherwise, matched either one another or the reference samples.

Clearly, there appear to be some issues concerning the suitability of the RAPD approach, as the aDNA samples used varied significantly, and showed no uniformity with one another or the control animals, and subsequently there appear to be some critical flaws concerning the use of RAPD when applied to samples from this site. These are considered below.

Background Contamination

In RAPD, negative controls did not always give negative results, as the *Taq* polymerase and Stoffel fragment have, like most *Taq* polymerases, a degree of contamination by DNA arising from their purification, and although not an issue in normal PCR, because of the low stringency of the PCR reaction, the negative control can yield a bacterial banding pattern. This does not affect normal RAPD PCR reactions, but the aDNA fragmented sequences may face competitive inhibition by this contaminating sequence. Is this significant enough to alter specific banding patterns? The bacterial contamination can be addressed, as this is reproducible bacterial banding (from the same bacterial source) and can always be detected. Unfortunately, the template competition on the other hand is not always so easy to detect.

Template Concentration

A number of studies have investigated RAPD variations, and have concluded that the RAPD technique is sensitive to slight modifications and band patterns may vary for a number of reasons, including handling (McEwan, *et al.*, 1998) and primer concentration (MacPherson, *et al.*, 1993). These situations can be overcome by strict adherence to established PCR protocols and laboratory procedures. Unfortunately, RAPD banding patterns also vary because of inconsistent template quantities (Davin-Regli, *et al.*, 1995), and different methods of DNA preparation (Tseng, *et al.*, 2001). aDNA varies greatly from one sample to another,

particularly in terms of condition. Two samples containing the same amount of DNA may differ in the amount of viable template DNA which may be amplified. Although quantification of DNA is possible in most cases aDNA cannot be quantified in terms of template. For example, the Towyn-Y-Capel samples produced no positive results with the Quantiblot human DNA hybridisation kit, even though DNA was present. Without a set amount of template, a reproducible banding pattern cannot be guaranteed, and this effect was verified by modern human DNA dilution series using RAPD (Figures 4.26-4.32).

Multiple Sequences

The RAPD reaction cannot differentiate between different templates (initially addressed above). This poses a serious problem with aDNA investigations due to the impure substrate where aDNA is found. aDNA is usually contaminated with a myriad of other DNA sequences (bacterial, fungal, plant and animal). These sequences are extracted with the aDNA, and the RAPD PCR cannot distinguish between them. Furthermore, due to the poor quality template of aDNA and the non specific annealing temperature, even minute amounts of good quality bacterial DNA may be amplified in preference to the aDNA template. This results in either the production of additional loci (yielding a multi-species banding pattern) or competitively inhibiting the aDNA amplification. This point has already been noted in previous studies regarding the use of RAPD in population studies (Isabel *et al.*, 1999), where even modern DNA species fingerprinting can be misrepresentative.

Absence of a Reference Databases

The RAPD technique is mostly used for small intrapopulation studies and, despite the extensive use of the technique in a variety of fields, no species databases have been established. This could be due to the variation in the 10-mer primer sequences used, or result from the large variation of the results between laboratories. Considering the dynamic differences between species, and reflecting on the number of species present, a reference database for comparison is highly desirable. Unfortunately, due to the fragmentary and non-specific nature of the loose find bones from Towyn-Y-Capel, morphological evaluation and hence species identification was not possible.

RAPD Conclusions

Although the RAPD method may be the method of choice for population or mutation analysis, it is a far too specific, sensitive and stringent for effective aDNA analysis.

Therefore, the results of the RAPD PCR experiments although successful in producing profiles, can be limited due to the reasons outlined in the previous section. However, the procedure was useful in the course of this investigation for the following reasons:-

I. It allowed effective comparison of extraction techniques and samples (DNA quantity) from different unknown species.
II. It shows that this technique is unreliable for species identification, despite its utility in this area (Lee & Chang, 1994).
III. The intensity of amplified DNA bands can be compared to bands of known DNA quantities in the DNA ladders used as molecular weight markers.
IV. The RAPD reaction was not noticeably affected by inhibition, possibly due to the dual enzyme activity and or the low annealing temperature of the RAPD PCR. Consequently, as a preliminary screen for cross species samples, it was effective within limits.

6.3.3 The Amplification of the Hypervariable mtDNA regions in the Towyn-Y-Capel Population

The ideal target for aDNA amplification is mtDNA, and as such is often the standard for aDNA investigations. For this research two sets of second generation mitochondrial primers were selected. These consisted of HV 1 and HV 2 specific multiplex sets of primers that are able to amplify multiple loci within these regions at the same time. This is a very effective method of screening, which provides information regarding the viability and condition of the DNA template, saves time and consumables and also reduces the risk of contamination. The design of these probes was such that they deliberately overlap and they are specific for the most polymorphic areas of the hypervariable regions.. The probes can be used for sequencing and, because of good fragment length differentiation, can be used for the more sensitive fragment length analysis (if primers are fluorescently labelled) using the ABI 310 gentic analyser to detect fragment size, inclusions and deletions.

The usefulness of the second-generation multiplex primers for low copy number research however was not as expected. The present results showed a considerable difference between the multiplex and a small target specific singleplex. The sensitivity or the ability to deal with inhibiting substances appeared to be far higher in the singleplex reaction. Inhibition played a part, as it prevented the amplification of up to half of the spiked samples in two independent amplifications.

This occurred despite the optimisation of the primers and use of various extraction techniques. Although initial surveys of random samples produced some significant positive results (Figure 4.38), subsequent experiments with human samples did not produce the predicted bands.

This was due to either the absence of amplifiable DNA or inhibition of PCR. Spiked samples contained known quantities of modern DNA to check for inhibition and half of these samples did not amplify a detectable product. This is clearly a consequence of PCR inhibition by the extracted material, and must be overcome for further PCR analysis. This may also be due to the choice of master mix ReddyTaq (commonly used within the School) which contains non-specified products that allow for direct loading into the gel. These products may either lower the sensitivity of the *Taq* polymerase or interact with the inhibitors by some undescribed mechanism. Five steps, therefore, were put into place in an attempt to overcome the inhibition:

I. The introduction of an extraction procedure designed to minimise soil inhibitors (§4.3.3),
II. The use of PTB (N-phenacylthiazolium bromide, reagent which cleaves glucose-derived protein cross-links)(§4.3.3; Poinar *et al.*, 1998)
III. The introduction of high quality second-generation *Taq* polymerase (§4.3.6) and further detailed optimisation of PCR through variation of parameters such as primer, extract concentration, volume and $MgCl_2$ concentration (§4.4).
IV. The use of PCR enhancers such as BSA, DMSO and Betaine, shown to minimise inhibition (Frackman, 1998).
V. Finally, some concern has been raised regarding the mtDNA primers used, specifically with regard to the large primer dimers formed. To rectify this situation new primers were designed in order to attempt amplification of alternative targets within the control region.

The Alonso primers, some of which were fluorescently labelled (A1F/A1R; 5F/5R), were introduced, along with the 4 other points mentioned, including increased lysis volumes to increase the gross volume of material processed and consequently the DNA recovered.

Unfortunately, despite RAPD results and some early successes no subsequent amplifications were successful (§4.3.5; §4.4.4). Given the degree of experimentation, the success of the RAPD showed a large degree of diversity and fragment size.

This was considered to be related to the endogenous contamination of the samples themselves which bares evidence in the high degree of aspartic acid racemization (§3.4). The DNA in the samples was therefore suspected of being too fragmented and or too degraded to amplify successfully, and consequently to sequence and retrieve useful genetic information.

Surprisingly then, independent samples, extracted and amplified at the paleoDNA laboratories returned successful and partially successful sequences for nine of the ten samples submitted (§5). The issues that arise from these sequence and the analysis are discussed further in the following section (§6.4).

6.4 Sequence Analysis and the Phylogenetic Reconstruction of selected Towyn-Y-Capel Individuals

Sequencing aDNA is unlike most other forms of genetic research, in that the sequence results require more stringent interpretation, as, most often the sequences obtained are interspersed with a variety of miscoding lesions, areas of poor amplification and base dropouts, which can only be resolved by multiple runs. Even then, the assembly of sequences takes on the distinctive form of a jigsaw puzzle, which involves careful attention to minute details of the information and assemblage. It is important, therefore, to consider what indicators constitute an authentic sequence (Topf et al., 2005).

6.4.1 Authenticity of the Recovered Sequences

The authenticity of recovered sequences is a concern in any aDNA research project. For the sequences to be considered as authentic they must meet a variety of conditions, the first of which is independent conformation by an external laboratory. The issue lies firmly in the fact that not only are the sequences not confirmed by this criteria, but are potentially contradicted by the negative results achieved at the UCLan laboratories. It is on this basis that the sequences recovered must be considered with some degree of scepticism. This not to entirely discount the authenticity of these sequences, and there are plausible reasons that might explain why onelaboratory onlt was successful. For instance, the cycle profile used at the PaleoDNA Laboratory was out of the range used at the UCLan due to the laboratory changes and background limits of the DNA. Essentially amplifications in excess of 40-45 cycles of human DNA are affected by “normal” background level of human DNA present in the laboratory, in the form of dust and aerosols and are therefore easily contaminated. It must be noted that conservative trials at 45 cycles completed at UCLan also failed to amplify aDNA from the Towyn-Y-Capel population, and all this despite the successful RAPD profiles of the

arbitrary sequences within the Towyn-Y-Capel samples. PaleoDNA facilities however, dedicated to aDNA and trace DNA research have a comprehensively equipped with positive pressure and dust free laboratories (clean room) with full UV lighting, changing rooms (to avoid bringing in dust), and full body protection (including face and shoes) in order to reduce background human DNA levels, and thereby allow multiple thermocycler profiles of 50 and over for human DNA.

It is significant, with regard to the sequences themselves, that they show all the hallmarks of aDNA sequences, miscoding lesions, base dropout, low overall peak height, rapid deterioration in quality with fragment size and high background levels.

The polymorphisms between the samples vary, although there appears to be a few running profiles amongst them, particularly the K haplotype mutations at T16224C, T16311C and C16320T, although these are interchangeable between the samples and in some cases are VPT that may be a result of a coincidental miscoding lesion. The main issue regarding these mutations is that they correspond to the key investigators HV1 haplotype K1a1a, although none of the sequences corresponds exactly. As to external contamination, i.e. from undetermined sources, there are some sequences that appear to exhibit consecutive mutations that do not match known modern haplotypes; but rather they exhibit the mutations of two independent haplotypes, which raises questions regarding contamination, although they may be miscoding lesions or an unusual haplotype. As to the origin of the sequences, these are consistent with modern European HV1 haplotypes, and there is no reason to exclude them on that premise although further discussion will be given in § 6.4.2-§6.4.4 with regards to the specific nature of the polymorphisms.

6.4.2 Correlation of Haplogroups & Sequences

Discounting the authenticity issue, the independent sequences were compiled into consensus sequences (§5) that were compared to the rCRS as shown in table 5.8, which includes the variable mutations, the most likely effect of the depurination and deamination. The different behaviour of these transitions was recorded as either VPT1 or VPT2 as defined in §5, which assists in estimating the authentic sequence, and which is a new approach to aDNA miscoding information. Normal mutations formed a basic sequence template or consensus and all VPT were considered as variant sequences and analysed independently, such that if there were 2 VPT mutations, there would be 4 possible sequences. Then the sequences were compared against three different populations in order to determine the homology to reported and or published sequence variations to deduce the likely haplogroup (Table 5.9).

This was in addition to looking at the basic haplogroup mosaics or defining mutations (Table 5.10) and a qualitative comparison and analysis of the consensus and variable sequences to the largest open access mtDNA haplogroup database (Behar et al., 2007) (§ 5.4).

The mutations themselves were compared with recorded mutations in order to understand the mutation and its normal associations with other mutations and modern haplogroups (Table 5.10). This provided a basis for the qualitative approach to determining the most likely haplogroup, or haplogroups the sequences belonged too, and therefore not automatically discounting possibly important mutations, and losing important resolution because of miscoding lesions. The conclusion of these observations was the ability to provide a finer resolution to the HV1 haplotype.

The mutations, VPT's and estimated haplogroups are summarised in table 6.2. An interesting observation, particularly looking a Sk56 is the presence of miscoding lesions or VPT's at sites that correspond to the defining mutations of not one haplogroup, but two independent haplogroups, which most likely indicates contamination or background amplification, a problem identified previously as a result of extreme profiling. The overall haplogroup determination does, however, match with European haplogroups, particularly with regards to the consensus. A further exploration of the intrapopulation distribution and comparison to modern and ancient European populations is given in the following section (§6.4.3). The greater extent of mtDNA haplogroup distribution throughout Europe can be seen in figure 6.1, although ratios are relatively homogenous, dominated by the H haplotype.

6.4.3 Phylogenetic Reconstruction

The limited number of sequences recovered made phylogenetic reconstruction difficult from the perspective of the intrapopulation variation and the significance to the modern haplogroup distribution, which is why a more qualitative approach has been used. The phylogenetic reconstruction using the Median-Joining Network Algorithm to construct a phylogenetic tree (Figure 5.5), the consensus sequences only were used in order to not be unrepresentative and because open base calls (Table 5.8) are not supported and the resultant data would be misleading unless coupled to all the sequence variations, which would be yield an unfeasibly large number of divergent and unrepresentative phylogenetic trees. Unfortunately, the small sample size means that no major conclusions can be drawn from the data apart from the mutual divergence of the Sk35, Sk41, Sk21 and Sk33 from a common mutation.

Table 6.1: Distribution of the Predominate Haplogroups in the North Atlantic Region Compared to Ancient Populations.

Country	H	V	J	T	U	K	I	W	X	Other	Set#
England[a]	39.5	3.5	9	12	15	9	3	2.5	2	4.5	1577
Finland[a]	40	6.5	5	4	**25**	3	4	**8**	1	3.5	580
France[a]	40.5	2.5	6	11.5	18.5	8	2.5	3	1.5	6	878
Germany[a]	40.5	4	8.5	10.5	15	10	3	2	1.5	5.5	2610
Iceland[a]	46	1.5	13	10	12.5	7.5	4.5	0	1.5	2.5	511
Ireland[a]	37.5	4.5	10.5	11	15	11	3.5	2.5	1.5	3	1397
Scotland[a]	42.5	3.5	14	11.5	13	6.5	**5**	1	1.5	1.5	1413
Spain[a]	40	5	4	6	11	5	2	2	2	23	498
4th Century[b]	N/A	6.5	6.5	16.1	22.6	-	3.2	6.5	3.2	N/A	31
11th Century[b]	N/A	-	11.8	35.3	5.9	5.9	-	-	5.9	N/A	17
Towyn-Y-Capel	22	-	-	-	22	55	-	-	-	-	9

This table lists the percentage of each of the top 9 haplogroups in each of the 8 countries listed from a selected sample set, the number of which is shown in Column (Set#). These results are further compared to two Early medieval European populations and finally with the "definite" Haplotype determined from HV1 results from Towyn-Y-Capel. [a]from Genebase Database (www.genebase.com); [b]Topf et al., 2006

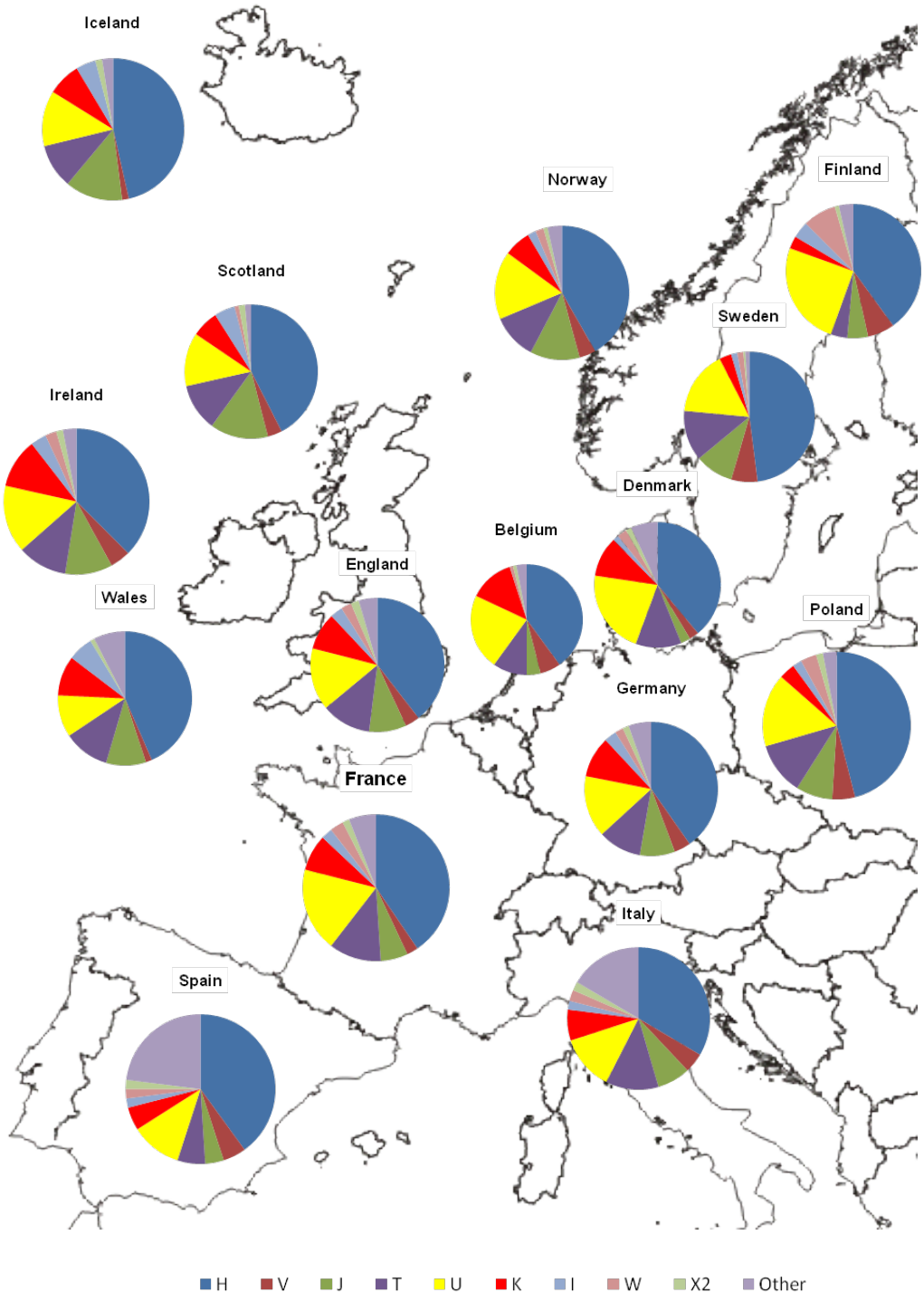


Figure 6.1: Haplogroup Distributions within Western Europe.
This is a visual representation of the percentage of each haplogroup found in sample sets from each country (Information from Genebase Database www.genebase.com).

6.4.4 Genetic Determination of Towyn-Y-Capel & the North Atlantic Populations

Although definite conclusions are hard to draw from the intra-population analysis due to the small sample size, observations can be made from comparison with larger modern and contemporary populations. Table 6.1 shows the modern haplogroup distribution in the North Atlantic countries compared to two ancient populations from England and the Towyn-Y-Capel population. Clearly, the diversity is limited, particularly at the haplogroup family level rather than the specific designations of the subclades, however, it can be clearly seen to be over represented by the K haplogroup, which is found in its highest modern percentages in Ireland, Germany and England. Interestingly, the haplogroup U distribution matches with the early 4th Century AD population distribution, which in turn is most closely matched with that of Finland. However, the specific information regarding distribution remains in the suborder of the haplogropups (or subclade) and their more recent distribution within the North Atlantic countries which unfortunately is very difficult given the degraded DNA of the ancient samples sequences..

6.5 Molecular Prospecting

As seen in Chapter 4, molecular prospecting is a term used to describe analysistaken outside of the confines of genetics, mainly using molecular chemistry this science looks into the condition of the greater organic faction of the calcified tissues, such as the building blocks of the collagen component, amino acids, and also the inorganic faction of samples in terms of the trace element profile and stable isotope ratio of strontium. The description is given in greater deal in the introduction (§1.33-1.36), and the results obtained by this approach are given in chapter 3. Overall, these investigations were, from a molecular perspective, the most enlightening, and given greater funds, experience and the availability of equipment, greater sampling of the population would have been carried out.

These investigations, given careful consideration early in the research and continually during sampling and storage, enable the maximisation of information from a selected sample or population.This also provides a perfect compliment to aDNA research, where the same source material may be used and segregated at the sampling stage.

6.5.1 Benefits of the Multidisciplinary Scientific Approach

It is in this section that threads of the taphonmic and genetic research elements are drawn together with the corroborative screening and information experiments of the organic and molecular analysis covered in chapter 3. Table 6.2 shows the mutual anthropological, taphonmical, molecular chemistry and genetic results from ten skeletons selected from across the skeletal population and archaeological stratigraphy (chronologically oldest, central to youngest burial sequences). The analysis concentrated on the molecular aspects and how they impact on the other factors.

Amino Acid Racemization

Amino acid racemisation, of aspartic acid in particular, is useful in evaluating the genetic potential of a particular sample. It is one of the best screening techniques for evaluating the condition of the biological component of teeth. As such, it is considered as a litmus test by many aDNA researchers (Poinar et al., 1996; Johnson & Miller, 1997; Krings et al., 1999; Thomas et al., 2003; Collins et al., 2009).

There is, however, some contention between researchers regarding the aspartic acid ratio limit as it applies to aDNA (§1.3.6), and whether it should be set at 0.10 or 0.08. According to the divergent genetic results, essentially the UCLan laboratory results, this study supports the 0.08 limit, as most of the samples analysed fell between 0.08 and 0.09. However, sequence results from paleoDNA laboratory do not. As shown in Table 5.2 complete HV1 sequences were retrieved from amino acid ratios above 0.08, but below 0.09, which would support the 0.09 ratio limit. The sequences samples also support that observation. It also true that the low amino acid ratio does not guarantee aDNA results, as shown in Table 5.1. Sk57, for instance, has a relatively low ratio at 0.067 but a low success with regard to sequencing, as only a partial sequence was recovered from ten sequencing runs (Table 5.8). Clearly, the amino acid ratios themselves may be contaminated, but that was considered in various ratio and concentration experiments performed on the samples (§3.4.1) which did not highlight Sk57 as divergent.

From an aspect of interest regarding the further applications of amino acid racemization as a dating technique (Ohtani & Yamamoto, 2005; Johnson & Miller, 1997), the Towyn-Y-Capel samples do not show any particular chronology associated with the aspartic acid racemization. Samples higher in the archaeological burial sequence (Sk21) have high ratios that are similar if not higher than those that are lower in the in the sequence (Sk51,Sk52 ,Sk56 & Sk60).

Although this may reflect the different rates of biological degradation due to varying environmental factors at the archaeological site itself and not the dating technique (Table 6.2). The skeletons recovered higher in the sequence for instance, may have been under greater degradative stress than lower sequences. Some evidence that may indicate this is that the lowest ratios were recovered from skeletons (Sk33 & Sk57) in the central sequence. The numbers within the population make the results difficult to verify. This is a common issue with ancient biomolecule research where the samples dictate the statistics. Within the population studied there does not appear to be any further correlations regarding the autofluorescence (QLF) or the preservation and condition of the skeleton or tooth (Table 6.2).

Strontium Isotope Ratio

As previously described (§1.34-136), the strontium ratios are indicative of teeth of childhood geographical origin, and along with the study of the local and regional geochemistry, can give an indication of the individual's migration during life. The range of strontium ratios recovered from the subset of the Towyn-Y-Capel population analysed were not only very high, but also diverse (§ 3.4.2). According to Evans (2006), these are perhaps the widest ranging set of strontium ratios sampled within Britain. By far, the single the most unusual of the ratios, was recovered from Sk56. Sk56 was anthropologically identified as a male individual from the lower archaeological sequence with an age of 50-60 years (Table 6.2). The remains were in very good condition, although the autofluorescence (QLF) of the teeth was at the lower end of the range, indicating some decalcification or diagenetic modification of the hydroxyapatite structure. The biological faction was in a similar state as indicated by a high AAR ratio.

The strontium ratio was unusual in that it indicates very strongly that the individual spent late childhood in a location of very young, typically volcanic rocks, , which are best represented in North Atlantic Europe by Iceland (Table 6.3, Figure 6.2). This is a very firm indicator of Scandinavian origin, which, coupled with the genetic analysis, also supports that theory that the haplogroup determined from the sequence shows defining mutations for the haplogroup K (Table 5.31), a subclade under haplogroup U. Both found in high proportions in the North Atlantic (Figure 6.1 and Table 6.1), particularly in Nordic countries and outlying islands of north and western Scotland and less commonly found in the remainder of modern Europe. Unfortunately, the main mutations are VPT's and include two mutations that are defining mutations for the haplogroup T (§ 5.8), which may be either contamination (or vice-versa), coincidental miscoding lesion, unique mutation, microheteroplasmy or a combination of any these events. The SNP that is verified however does support the haplogroup K designation, as a defining mutation (§5.8)

Table 6.2: Results of the Multidisciplinary Analysis of Towyn-Y-Capel Skeletal Teeth													
Anthropological Analysis				**Taphonomical Analysis**			**Chemical Analysis**		**Genetic Analysis**				
Sk#[1]	**Preservation[2]**	**Burial Site Position[3]**	**Age[4]**	**Tooth[5]**	**GPI[6]**	**QLF/ %QLF[7]**	**AAR[8]**	**$^{87}Sr/^{86}Sr$ Ratio[9]**	**HVR 1 Variations [10]**			**HV1 Haplotype[11]**	
									SNP	**VPT 1**	**VPT2**	**Likely**	**Possible**
Sk 21♂	Moderate	Higher of burial sequence (Later)	Adult	RM^3	2	66.95/68%	0.084	0.7159	C16222T T16224C C16270T	C16052Y	T16311C	**K1b**	U5 (U5a) T*(T3)
Sk 33 ♀	Moderate	Centre of burial sequence 1270+/60 (AD 650-890)	20-30	RM_3	2	78.85/80%	0.066	0.7179	A16212G C16222T T16224C C16270T	C16021S	T16311C	**K 1b**	H* U5 (U5a)
Sk 34♂	Good	N/A	35-45	LM_3	3	124.3/126%	0.083	0.713	C16182T C16192T C16270T	A16252R	G16049R C16130M C16221Y C16278Y T16311C	**U***	**(U5a/b2)** H 21 (H11)
Sk 35 ♂	Good	N/A	15-25	LM_2	2	85.80/87%	0.07	0.7103	None	None	T16311Y C16320Y	**K***	**(K1c1 or K1c2)**
Sk 41 ♂	Good	N/A	30-45	RM_2	3	69.67/71%	0.081	0.7155	T16224C	None	None	**K***	H*
Sk 51 ♂	Good	Lower in burial Sequence 1180+/-50 (AD 710-980)	30-49	LM_3	2	83.86/85%	0.076	0.7148	G16329A T16342C A16343G T16356C	C16295Y	None	**U***	**(U3-4)**
Sk 52♀	Moderate	Lower in burial Sequence	25-45	RM_2	3	70.70/72%	0.075	0.7095	None	None	None	**H***	
Sk 56♂	Good	Lower in burial Sequence (Earlier)	50-60	LM_3	2	71.81/73%	0.082	0.7058	T16224C	None	C16294Y C16296Y T16311C C16320Y	**K 1a1a**	T*(T2)
Sk 57 ?	Moderate	Centre of burial sequence Partial Skeleton	25-35	RM_2	2	83.96/85%	0.067	0.7146	None	C16218Y C16228Y C16250Y A16289M	None	**H*[12]**	**(H2)**
Sk 60 ♀	Good	Lower in burial Sequence	35-45	LM_1	N/D	133.3/135%	0.082	0.7181	N/A	T16330K	N/A	**N/A[13]**	**N/A**

This table shows the comparison between the final results from the anthropological analysis (§2), taphonomical analysis (§2), chemical analysis (§3) and genetic analysis (§5) for the ten samples listed in the first column (Sk#). From this table the information and relationships between the sample, its organic and inorganic preservation, strontium ratio and likely haplotype can be deduced. [1]Archaeological skeleton designation & estimated sex determined by anthropological analysis (§2.2.3); [2]Overall skeleton preservation (§2.2.3); [3]Estimated age determined by anthroplological analysis (§2.2.3); [5]Designation of sampled tooth (§1.3.3); [6]Gross preservation index, (1- poor, 2-Moderate, 3-Good, 4-Excellent § 2.3.2); [7]Quantitative light fluorescence determined from occlusal suface only/% integrity compared to control (§2.4.5); [8]Amino Acid Racemization, ratio's above 0.08 considered unlikely for succesful DNA recovery (§ 3.4.1); [9]Strontium isotope ratio $^{87}Sr/^{86}Sr$, expected local Range 0.711-0.716, Human control 0.7139, Animal control; 0.7124, Ocean water 0.7092 (§3.4.2); [10]HV1 mutations from reference sequence (rCRS) in the sequenced regions of rCRS positions 15972-16422 (§ 3.31); [11] Haplogroup estimate from HV1 mutations, favourable haplogroup detemination shown first in bold as Likely followed by possible determinations. [12] Partial sequence [13] Fragmented Sequence.

The remainder of the samples are within the expected range given the local controls and the uniquely diverse local geology. The female Sk52 of middle age, with a low ratio, may come from South Wales or Britain, due to the lower strontium ratio that corresponds to the cretaceous chalk of the greater part of the UK. This is supported somewhat by belonging to the more common and widely distributed H haplogroup as extrapolated from the H haplotype (Table 6.2),. At the higher end, possibly too high for the surrounding geology (0.7159-0.7179), two adult skeleton samples, one male one female, Sk21 & Sk33, are of interest., Sk21 was higher in the archaeological sequence than Sk33 which had a carbon date of between AD 650-890 (Table 6.2) and of moderate skeletal and tooth condition and low QLF, with diverging AAR ratios, one high (Sk21) whilst the other a lower ratio (Sk33). They stand out as both share distinctive and defining mutations for the K1b subclade of the K haplogroup, although Sk33 has a unique mutation at A16212G. As to the high strontium ratio, it indicates that they come from a very old geological area, perhaps older than the late PreCambrian (or Proterozoic rocks) of the Anglesey area. Other areas with old Geology include the North Western Isles of Scotland and some coastal areas of North Eastern Ireland though the geological age is similar to Anglesey. Southern Norway also has high ratios so is a remote possibility along with the even more remote Greenland, which although having some of the oldest Geology in the North Atlantic is an unlikely source due to the sporadic Scandinavian settlement.

6.5.2 Trace Metal Analysis of Towyn-Y-Capel Population

The study introduced the use of more sensitive ICP-MS technology, namely MC-ICP-MS, which can be used for highly sensitive strontium isotope ratio determination in place of the TIMS and has a number of advantages over the older technique (Hoddell et al., 2004). These include the simultaneous discernment of the strontium isotope concentrations, alongside a more general trace element concentration determination for the same sample. This was primarily conducted to verify the accuracy of the technique relative to the strontium concentrations through the detection of anomalies. However, the individual skeleton element ‘profile’ can more significantly be used to ascertain the health and dietary status of the individual. As shown in chapter I (§1.3.4) trace elements may indicate a variety of dietary or environmental conditions from Roman lead poisoning (Whittaker & Stack (1984) to nutrient deficiency and high fibre diets. It also must be considered that the samples selected, being part of the enamel from 2nd and 3rd molars, represent the diet during mid to late childhood. The results in chapter 4 generally show that the individuals were in good health, compared to modern day individuals the levels were low, except for magnesium, and manganese, though even these elements were within the healthy range (Table 3.2). The low levels are consistent with a non heavy metal polluted pre-industrialised society and are therefore representative of expected results.

Geographical Area	Predominant Rock	Range of $^{86}Sr/^{87}Sr$	Reference
Iceland	Volcanic Bedrock	0.7056-0.7096	Dickin, 1997
Northern Ireland	Tertiary Basalt	~ 0.7070	Price & Gestsdottir, 2006
Ocean Water	N/A	~ 0.7092	Price & Gestsdottir, 2006
England	Cretaceous Chalk Triassic Sandstone	0.7080-0.7120	Budd *et al*., 2000
Orkney Islands	Red Sandstone	~ 0.7123	Montgomery *et al*., 2003
Anglesey	Scheists & Gneisses	~ 0.7130	Davies, 1983
Southern Norway	Gneisses & Granites	0.7087-0.7185	Aberg *et al*., 1998
Greenland	Ancient Metamorphic rock	0.7250-0.7550	Hoppe *et al*., 2003

Table 6.3: Geographic Range of Strontium Ratios in the North Atlantic
Basic outline of recorded $^{86}Sr/^{87}Sr$ of the Northern Atlantic Rim. Values are representative of the strontium in the local geology, biologically available strontium ratios may vary, normally a bit lower than the surrounding geology.

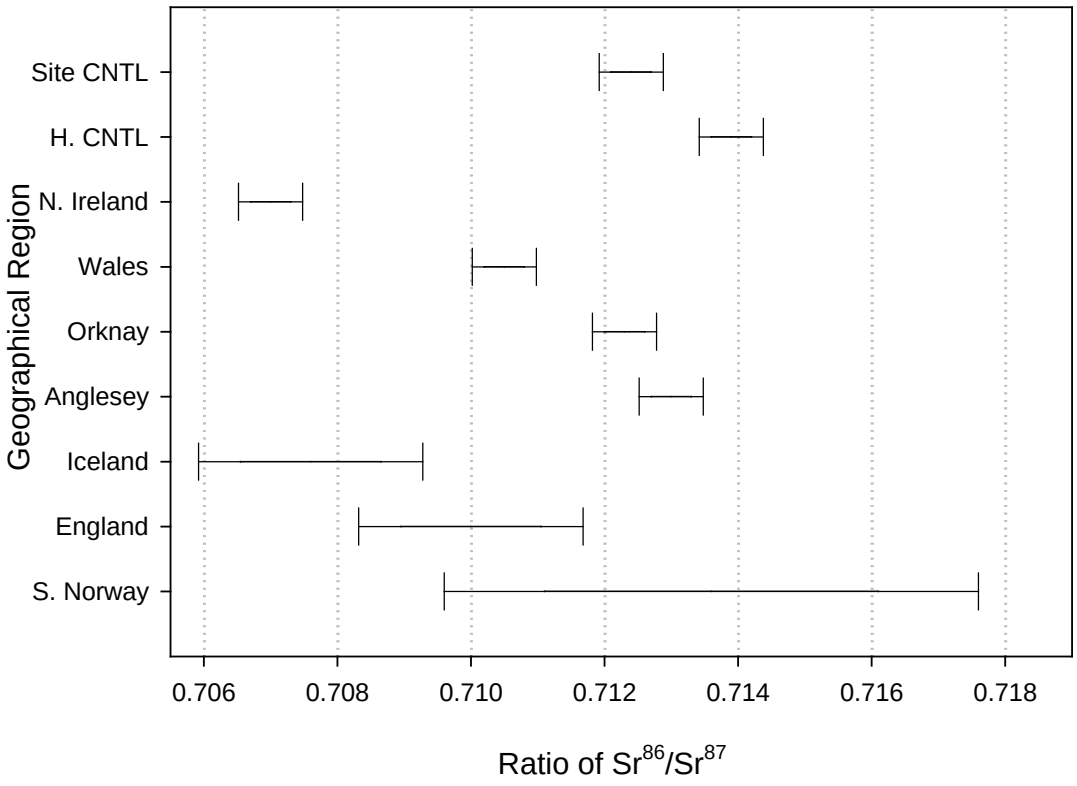


Figure 6.2: Regional Strontium Variations in the North Atlantic Rim Area.
Bar graph compares the range or spread of strontium ratios (Table 6.3) compared to Towyn-Y-Capel (ancient horse tooth) and Anglesey control samples (modern human tooth).

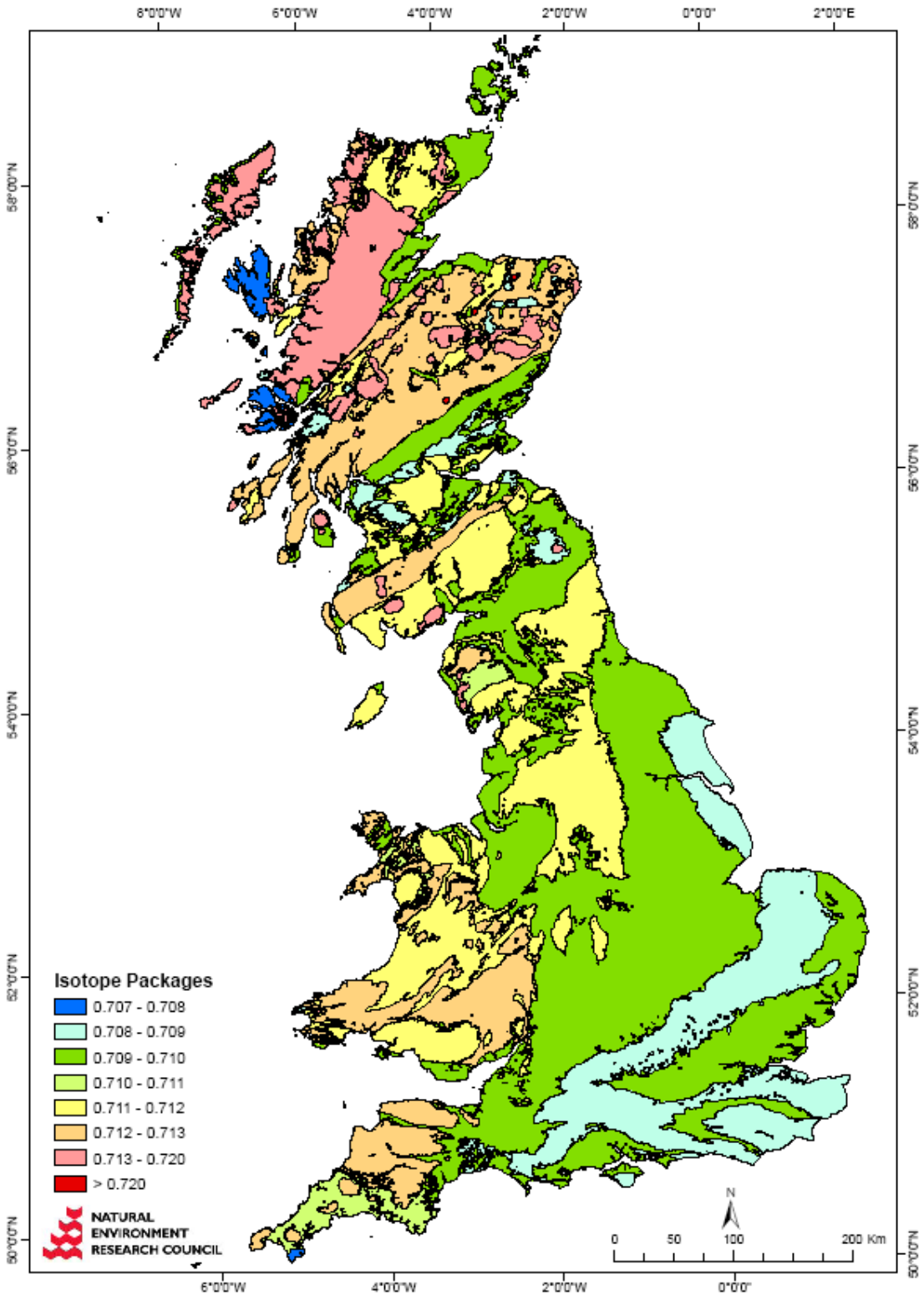


Figure 6.3: Biologically available strontium isotope distribuition in Britain.
Strontium isotopes in Britain normally vary between 0.7080 and 0.7100. The rock formations around Anglesey are particularly old and likely to give higher ratios than the surrounding area. There is a compositional gap in the averages between 0.713 and 0.716 at one sigma confidence levels which suggests that averaged values within this range should be uncommon in Britain. Adapted from Evan et al., 2010.

Overall, no great anomalies were found in the trace elements assayed, either as toxic or deficient levels. On the individual basis and within the population itself the levels of manganese and iron were abnormally high for Sk41 (Figure 3.6 & 3.7) which may indicate that this individual had a different diet than the remaining population, as both elements are essential (§1.3.4; Tables 1.8), possibly higher in meat. An unusually high level of lead, compared to the rest of the population was also found in one individual (Sk56) (§3.4; Table 3.11). This may indicate exposure to low levels of environmental lead conditions different from the remainder of the population. This was possibly due to higher lead levels in the diet due to elevated levels in the surrounding geology, which is particularly important since it coincides with the low strontium isotope levels and the implications regarding an Icelandic origin (Table 6.2; Figure 6.2).

6.6 Novel Techniques, Trial Experiments and Forays into New Technologies

During this research, a number of new techniques and applications were used to improve the understanding of the diagentic status of the ancient remains and the status of the DNA within. One technique that showed particular promise was the combined use of the QLF and X-ray transmittance as non-destructive screening tools for the analysis of the tooth condition and state of tissue decalcification. These were particularly useful techniques, particularly in readdressing microvolume decalcification of aDNA extracts (§4.4.1) and assessing the inorganic structure of teeth (§2.3.3; §2.3.5). This technique, despite increasingly popularity in dental research, is limited by the poor understanding of the fluorescent source and its intimate association with the calcium in the tooth. Further investigation into the nature and identity of the flouresent molecule is essential to take this technique further forward for this application.

6.7 Final Considerations

A multidisciplinary approach to the recovery, handling and molecular study of archaeological remains enables a wider envelope of understanding and information retrieval, which is demonstrated by this research. Ancient DNA research provides a number of challenges that need addressing. Often, undertaking a comprehensively diverse approach is the best, particularly given the low success rate of even the most successful analysis. This is particularly important when coupled with the inhherent variability of archaeological sites, the affect of localised microenvironments, individual skeletal assemblage and the need for independent conformation required for authentication.

Taphonomy is clearly an important aspect of any comprehensive study on ancient remains, to ascertain anthopological information, to record data before destructive analysis, and is compatible with particular sensibilities with regard to the treatment and fate of human skeletal material. Observations on the Towyn-Y-Capel samples at gross, cellular and structural levels indicated that samples were moderately to well preserved which reflects the overall skeletal analysis. As a screening technique, gross morphology is clearly useful, though qualitative rather than quantitative, autofluorescence, which principally applied to tooth sample but can also apply to bone, provides another level of insight into the gross morphology of the tooth structure itself, which can take on a quantitative aspect though requiring some modification of currently available technology. Histological approaches can provide further specific information, but should be avoided where there is limited or sensitive material as the information ascertained does not compliment the research to that degree, which is why most of the histological research undertaken used loose animal finds to gain insights into the cellular condition and the degree of infiltration and diagenetic damage.

Clearly, for genetic research for molecular research, sample selection is imperative, and should not be influenced by availability or anthropological importance, but purely by the research goals with particular attention to the material itself. Cortical bone or teeth provide the only samples worth investigating in aDNA, preferably teeth, as demonstrated in this research, which easily enables a coupling with complimentary molecular research.

From a genetic perspective, RAPD analysis clearly shows the diversity of genetic material present in the aDNA extract from the archaeological remains. However, the main component of the RAPD research failed to succeed. Species deferentiation within the genetic aDNA mix extracted from bones and teeth were unresovable with RAPD. Even when these were successful, results were not always reproducibly verifiable. This is a clear concern in aDNA research and should be expected, and where possible prevented, by appropriate preparation, not just in the experimental part but the availability of appropriate aDNA facilities. Even when this is met however and sequences successfully retrieved and analysed, the nature of the aDNA means the sequences will have the tell tale signs of the degraded nature of the genetic target, with signs of the typical C-T heteroplasmic behaviour of deaminated and depurinated DNA. Finally, there are implications regarding the small population size that make it difficult to ascertain given small samples sizes and the condition of the sequences.

Molecular techniques, on the other hand, normally provide a wealth of information regarding the diet and the migration of the individual in moderate to well preserved teeth, and in addition to providing good support as to the population movements. Future research should concentrate on these techniques as the basis for research into archaeological populations along side supplementary

DNA research, to provide a full molecular picture of the ancient population. The key difficulty therein is the appropriate treatment of samples as to allow later genetic amplification, or preferably simultaneous research.

6.8 Conclusions

The Towyn-Y-Capel population as sampled clearly demonstrates that good preservation of the material from an archaeological site does not necessarily equate with good biological preservation. Amino acid analysis clearly shows that the samples were very degraded, and near the proposed limit of successful aDNA amplification, which is borne out in the genetic results. The key points to address relate to the burial mound itself, being composed of sequential sand and humic soil layers, both of which are the source of issues with regard to the biological preservation of skeletal material. Sand is often associated with leaching, and humic soils are an excellent source of humic and fulvic acids that interfere with the PCR reaction.

The biological condition was not shown in either the gross or the histological preservation, which indicated that the diagenetic breakdown was not heavily mediated by micro-organisms, although other diagenetic activity is known to have chemical a chemical nature as well as the biological and physical. The availability of water, which may in this case be a key factor, with sandy soil, close to the sea and in an area with a yearly rainfall of 1000 mm that may be an issue. This is complimented by the normally alkaline nature of soil, although the soil surrounding the burials had a neutral pH (pH 7.13), which is favourable for preservation, alongside the moderate soil temperature of between 5 - 20^{o}C.

The inorganic condition was also affected, shown by the diverse range of fluorescence results in teeth, ranging not only lower, but higher than the control, which may indicate modification of the inorganic component through a process similar to remineralisation, that may explain the divergent results. Trace element analysis, however, does not support the effect to any large degree, as in most samples the soil concentrations were vastly different from the human samples, which generally showed homology.

The genetic condition is reflected in the biological results, although in RAPD, the diverse banding profile and large products indicated recent but not modern contamination. This was from a varied source within the cleaned bone and teeth itself and probably indicative of infiltration by microorganisms into the bone and teeth samples duing degradation. Noticably, exogenous DNA presence was proportionally lower in tooth sample compared to bone samples. Pre-PCR DNA

quantification using thin microgel horizontal electrophoreses and time exposure CCD (sensitive to 600ρg) and picogreen fluorescent DNA detection did not detect any DNA.

Evidence using spiked samples show that some bone extracts were being inhibited, despite the fact that no observable stain or fluorescence indicative of humic substances was detected, which may indicate the DNA was inhibiting itself through the presence of Maillard products. PTB was added to the solution in order to overcome this with little success. A number of other techniques were attempted to improve the decalcification, extraction and purification of the DNA extract, optimisation of the PCR reaction and the use of PCR enhancing additives without success.

Independent verification of a population subset however was more successful, retrieving 6 complete sequences, and 3 partial sequences of varying quality. From these, it was ascertained that the population had the following haplogroups K, K1b, U^{*} (U5a/b2) and H*, which represents a rather modern haplogroup range.

Migration analysis determined through strontium analysis showed a diverse range of ratios achieved, which compliments the diverse geology of both Holy Island and Anglesey Island. The levels were predominately high, reflecting the late preCambrian-early Phanerzoic geological formations that dominate the geology of Holy Island. An outlier, however, was detected, with a very low strontium ratio, a ratio which is singularly representative of the geology of Iceland (Table 6.2 & 6.3). That indicates the unusual cohabitation of a Norse individual within the local area, at a time of regular Viking raids on local churches, although a recently excavated settlement at Llanbedrgoch (Redknap, 2000; pp 69) has indications of a Viking settlement, and it is well documented that the Kings of Gwynedd often fought alongside the Vikings of Dublin and the Isle of Mann.

Further work

There is a great deal of future work that has been touched upon in this research. QLF is for instance a particularly interesting area, specifically as we understand so little regarding the dynamics of the fluorophlore, let alone its interactions with the organic and inorganic factions. Research into identifying the molecule responsible for autofluorescence and its localization within the hard tissue matrix would be instrumental in further validating QLF not only for ancient DNA research, but for dentistry as well.

As to the chemical techniques used strontium analysis of the remainder of the population, given its success, in a more comprehensive study is clearly advisable, with greater funds would be recomended as the unique geology of the area compared to the surrounding does present a unique historical landscape.for elemental research and answering a unique historical questionwhat really was the early medieval population contact with the Norse peoples?

In addition more sensitive C:N ratio analysis, and possibly stable isotope ratio could assist in determining not only thee ancient population prevalent diet, but also the last movements before they died, specifically if we compare the Oxygen isotope ratios, for instance, between the femur, the tooth enamel and even the dentin.Given a large enough sample size and an abudance of local and contemporary bone samples, on a larger project multidisciplinary project, it might be possible to determine an individuals movements within the last 7 years (depending on the state of preservation of the sample as assessed by amino acid composition ratio and racemization. Indeed the future of archaeology science is to gain more information from less destructive sampling.

Another area that I think is of particular interest is an optimised form of *in-situ* DNA amplification and detection, techniques such a FISH (Fluorescent in-situ hybridization) for example. Deveoping a more sensitive/specific technique to amplify DNA sequences *in situ* without exposing the DNA to agreessive decalcification and isolation procedures may infact allow recovery of significant DNA amplicons from ancient material. It may even may revolutionise fields such as archaeogenetics and forensic genetics, where issues such as LCN-DNA, backround and lab contamination, may be avoided.

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