Population genetics of bottlenose dolphins (Tursiops truncatus) from an Anglo-Saxon archaeological site in comparison with modern populations.

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Courtney Dawn Nichols

Submitted for the Degree of Master of Science

University of Durham

Department of Biological and Biomedical Sciences

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Bottlenose dolphins (*Tursiops truncatus*) exhibit a cosmopolitan distribution, being found in temperate and tropical oceans throughout the world. Members of this species in the waters surrounding Britain are the most northerly populations and show a discontinuous modern distribution. The excavation of an Anglo-Saxon site at Flixborough, near the Humber Estuary in eastern England, has yielded the largest archaeological sample of bottlenose dolphin remains yet found in Britain, however, the estuary does not currently support a bottlenose dolphin population. This offers an opportunity to study the temporal dynamics of genetic structure for this species in a region where their conservation and management is of concern. Comparisons of mtDNA control region sequences and microsatellite genotypes from the remains at Flixborough with modern samples from elsewhere in the British Isles and around the world were completed. The results show that the samples from Flixborough form a genetically distinct population from all modern groups, including those around the United Kingdom and Ireland. It is suggested that a local population existed in the Humber Estuary during Anglo-Saxon times, which was most likely established through a founding event and has since become extinct. Possible causes of this local extinction and implications for management of modern populations around the British Isles are discussed.

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Table of Contents

List of Figures

List of Tables

Candidate's Declaration

I confirm that no part of the material offered has previously been submitted by me for a degree in this or in any other University. If material has been generated through joint work, my independent contribution has been clearly indicated. In all other cases material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

Statement of Copyright

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Abbreviations

aDNA	Ancient DNA
bp	basepairs
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotides
EDTA	Ethylenediaminetetraacetic Acid
HCl	Hydrochloric Acid
KCl	Potassium Chloride
M	Molar
$MgCl_2$	Magnesium Chloride
mL	milliliter
mM	millimolar
mtDNA	mitochondrial DNA
ng	nanogram
µg	microgram
µL	microliter
NaCl	sodium chloride
ng	nanogram
PCR	Polymerase Chain Reaction
TE	Tris-EDTA
UK	United Kingdom

Chapter I – Introduction

I.1. Tursiops truncatus (bottlenose dolphin)

I.1.1 Species Characteristics

Tursiops truncatus, commonly known as the bottlenose dolphin, is a small cetacean that inhabits temperate and tropical waters throughout the world. The order Cetacea includes all animals commonly referred to as whales, dolphins, and porpoises, and is separated into the families of Mysticeti (baleen whales) and Odontoceti (toothed whales). Most of the smaller odontocetes are popularly referred to as either porpoises or dolphins, and *Tursiops truncatus* belongs to the latter group.

The bottlenose dolphin is a primarily coastal species and is quite often seen in shallow bays and estuaries. Studies by dos Santos and Lacerda (1987) and Wilson et al. (1997) reported frequent observations in shallow waters near the mouths of estuaries, and shore-based sightings are frequent enough to have been used in several studies of United Kingdom (UK) populations (see Berrow et al. 1996; Wood 1998; Hastie et al. 2003). However, bottlenose dolphins can range further out into open waters (Kenney 1990; Wells et al. 1999) and distinct "inshore" and "offshore" forms have been identified in many parts of the species' range based on distinct genetics, morphometrics, and other biological parameters (Duffield et al. 1983; Hersh and Duffield 1990; Hoelzel et al. 1998; Kenney 1990; Ross and Cockcroft 1990; Van Waerebeek et al. 1990; Wang et al. 1999).

Few physical barriers to movement exist for species living in the marine environment, yet most studies of bottlenose dolphins have shown individuals and community groups with some definable home range (reviewed by Evans 1987 and Shane et al. 1986). Resident animals have been confirmed by re-sightings of recognizable individuals at locations in various parts of the world, including waters off of North

America (Scott et al. 1990), mainland Europe (dos Santos and Lacerda 1987), and the British Isles (Berrow et al. 1996; Grellier and Wilson 2000; Wilson et al. 1997). Nevertheless, even resident populations are not static and do exhibit shifts in the use of waters within their range. Wilson et al. (1997) reported seasonal movements and changes in numbers within animals in the resident population in the Moray Firth, Scotland, and Wells et al. (1990) observed the extension of a California resident population's range coinciding with an increase in water temperature. Moreover, long range movements of several hundred and even thousands of kilometers have been recorded for both individual animals and groups (Lockyer 1978; Wells et al. 1999; Wood 1998; others reports reviewed in Shane et al. 1986)

These movements may be associated with shifts in the distribution of prey, and seasonal migrations and shifts in numbers in an area are likely related to this (Shane et al. 1986). However, studies of stomach contents from stranded and by-caught animals show that bottlenose dolphins are able to exploit many different food sources (Blanco et al. 2001; Cockroft and Ross 1990; Santos et al. 2001). This ability could explain why some animals are able to show year-round residency when prey sources likely vary throughout the year. Still, Cockcroft and Ross (1990) stateed that it may be inappropriate to refer to the species as an opportunistic predator because they do exhibit clear preferences in prey. Many populations also show distinct, sometimes apparently socially learned, feeding behaviors used to exploit locally available or preferred food sources (Shane 1990). After a review of various reports of these specialized feeding behaviors, Shane (1990) suggested that this flexibility in feeding behavior has played a role in the bottlenose dolphin's success in diverse habitats.

The individual and group movements reported for bottlenose dolphins also imply that even resident populations may not be reproductively discrete, and as Shane et al.

(1986) concluded, the opportunity for genetic exchange between populations appears to exist. Scott et al. (1990) supported this with observations of schools in Sarasota Bay that contain dolphins from adjacent communities and exhibit relatively high levels of genetic heterogeneity, suggesting the resident community is not a closed reproductive unit. A lack of mitochondrial DNA (mtDNA) divergence among sympatric but socially separate populations in the Gulf of Mexico also implies reproductive mixing (Dowling and Brown 1993). However, genetic studies by Parsons et al. (2002) on bottlenose dolphins inhabiting Irish and British waters indicated significant differences in mtDNA between some putative populations around the isles, while Dowling and Brown (1993) also reported regional genetic differentiation along the southeastern Atlantic coast of the United States. It therefore seems that there are no definite trends for determining population divisions in this species, and this must be studied on a regional basis.

I.1.2 Inhabitants of British and Irish Waters

Bottlenose dolphins found in waters around Britain and Ireland inhabit some of the most northerly ranges for this species. They are common off the Atlantic coasts of Ireland, including a resident population in the Shannon Estuary (Berrow et al. 1996), but elsewhere in British waters they show a patchy distribution (Evans 1993). Resident populations also have been reported in the Moray Firth (Hammond and Thompson 1991; Wilson et al. 1997), the Sound of Barra in the Outer Hebrides (Grellier and Wilson 2000), Cardigan Bay (Grellier et al. 1995), and the Cornish coast (Wood 1998); however, sightings or strandings are rare outside of these areas (Evans 1993). Figure 1.1 shows locations for each of these populations. Notably, there is a lack of a resident population along the English North Sea coast.

Figure 1.1. Map showing locations for published accounts of resident populations of bottlenose dolphins in British and Irish waters. Populations are reported in the Shannon Estuary (Berrow et al. 1996), Moray Firth (Hammond and Thompson 1991; Wilson et al. 1997), Sound of Barra (Grellier and Wilson 2000), Cardigan Bay (Grellier et al. 1995), and off the Cornish Coast (Wood 1998).

Although individuals in these areas do show some degree of residency, they also show seasonal movements in distribution throughout their range and a peak in numbers during summer months (Grellier et al. 1995; Wilson et al. 1997). There are also reports of long-distance migrations for animals between Cornish and Welsh waters (Grellier et al. 1995; Wood 1998). Wood (1998) additionally reported transience in this species around Britain, as the presence of a resident population off the Cornish coast was newly reported in 1991 after an absence of resident animals in this area for a decade. No discussions of inshore and offshore forms have been published for animals around the British Isles and use of both inshore and offshore waters has been reported for individuals in the Moray Firth (Wilson et al. 1997) and Cardigan Bay (Grellier et al. 1995).

Studies of diet for animals in UK waters have only been published for animals in the Moray Firth. By examining stomach contents, Santos et al. (2001) demonstrated a fairly diverse diet consisting mainly of fish from 19 species, but also including cephalopods, along with crustaceans and polychaetes in a few individuals. Three types of fish, however, made up 77% of the estimated prey weight. This agrees with findings discussed earlier for populations in other parts of the world where, although catholic feeding habits were found, there was also a preference for certain prey items (Cockroft and Ross 1990). Additionally, seasonal variation in prey importance was found for UK animals (Santos et al. 2001), a likely adjustment to available prey sources. Evans (1980, 1987) reported that bottlenose dolphins in the UK feed mainly on inshore bottom-dwelling fish. Populations in the Moray Firth and Shannon Estuary show the highest abundance near narrow, deep passages where it is thought they can take advantage of strong tidal flows for foraging on this type of prey (Berrow et al. 1996; Hammond and Thompson 1991; Ingram and Rogan 2002; Wilson et al. 1997). Furthermore, Hastie et al. (2004) actually found a correlation of high intensities of feeding behavior with areas of great

abundance and steep seabed gradients for animals in the Moray Firth. However, other animals, such as those in Cardigan Bay, are reported to forage in all areas of their range (Grellier et al. 1995).

The small numbers of bottlenose dolphins in UK waters, along with their concentration primarily into resident, and perhaps reproductively discrete, populations, has yielded concern for the conservation of these animals (Simmonds 1994; Thompson et al. 2000). Much attention has especially been given in recent literature to low numbers along the North Sea coast of England and a possible contraction on this coast over the last 30 years (Evans 1980, 1987, 1993; Kayes 1985; Parsons et al. 2002). Stranding records kept by the British Museum of Natural History since 1913 (Harmer 1927; Fraser 1934, 1946, 1953, 1974; Sheldrick 1989, 1994), along with sighting records initiated in Britain in the 1970's (Evans 1980, 1993), are cited for this concern and allow an analysis of changes in populations over the last century.

I.1.3 Historic Distribution in British Waters

Stranding records indicate fluctuations in frequency of bottlenose dolphin strandings throughout the 20th century, but there are strandings reported for nearly all areas of the British and Irish coastlines over this time. Frequencies of strandings reported in Scotland and Ireland, however, are much lower than for England and Wales. The low numbers from Scotland may be due to differences in abundance, but they may also be influenced by the fact that these animals are not considered "Fishes Royal" in this country and strandings therefore, are not mandated to be reported (Evans 1980; Kayes 1985). This lack of representation in stranding records for Scotland is supported by Evans's (1980, 1993) discussion of cetacean sightings in which they are noted as being regularly reported off of northeastern Scotland. Care must also be taken in interpretation of stranding records

because correlations of stranding numbers with population sizes may not necessarily be straightforward as the reasons for strandings are not always clear, variations in coastal landscapes will affect the likelihood of any stranding being reported, and prevailing wind and water currents will determine if and where a deceased animal will be washed ashore (Kayes 1985).

Figure 1.2 seeks to address the concern about a decline of bottlenose dolphin numbers along the North Sea coast of England by showing the proportion of total bottlenose dolphin strandings from around the UK and Ireland that occurred on the English North Sea coast from 1913 to 1992. The first thing this figure clearly shows is that numbers of strandings on the North Sea coast have fluctuated throughout the century, ranging from 4% up to 47% in different decades. Likewise, records of cetacean strandings and captures kept for the coast of Denmark, which also give information about the state of these animals in the North Sea, indicate that their frequency has been variable since the 1600's (Kinze 1995). These historic sources agree with more current observations of shifts in populations around the UK such as the temporary absence of a population in Cornish waters during the 1980s (Wood 1989).

Figure 1.2 also supports the concern over declining numbers of animals on this coast in the last 30 years, with a drop to 12% or less of strandings occurring on the North Sea coast since 1970. Sighting records, even though they only began in the 1970s, additionally support a low number of bottlenose dolphins off this coast during these years, and notes from observers of more frequent observations of this species in previous decades also are reported (Evans 1980).

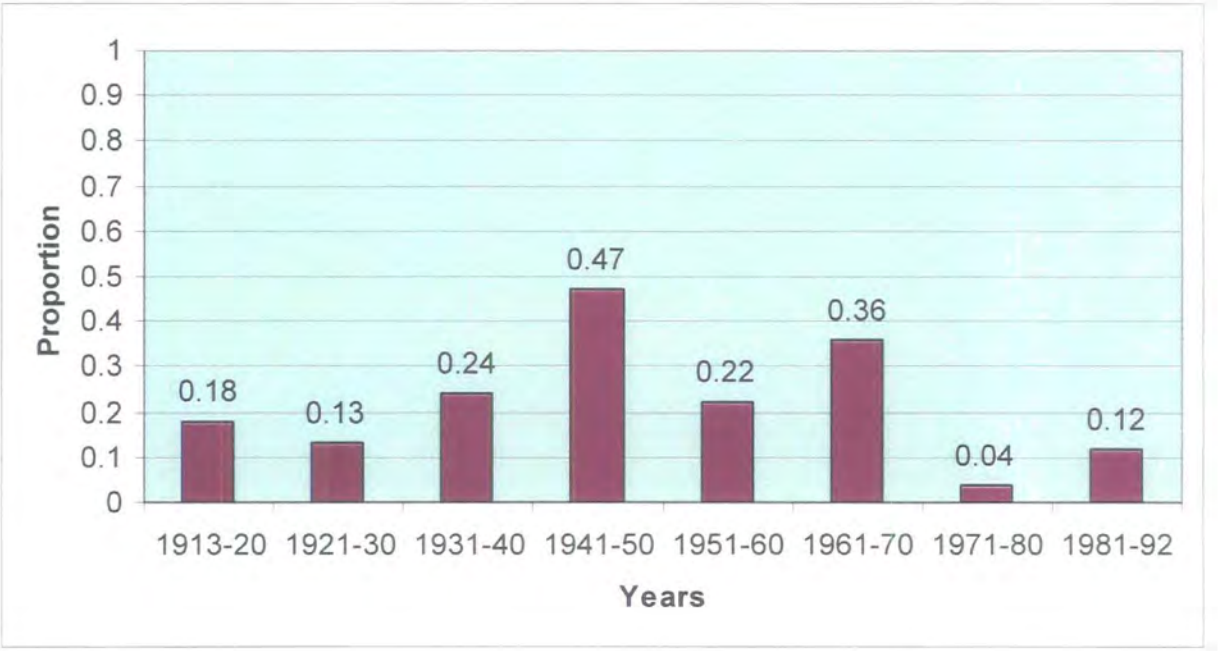

Figure 1.2. Proportion of total strandings from the UK and Irish coastlines, separated by decade, that occurred along the North Sea coast of England from 1913 to 1992. Data was taken from Harmer (1927), Fraser (1934, 1946, 1953, 1974), and Sheldrick (1989, 1994).

Figure 1.2 additionally makes apparent the low proportions of the total strandings that have occurred on that length of coastline during most decades. This is most notable when it is taken into consideration that strandings along the entire Scottish coast will be underrepresented because they are not required to be reported. This means that nearly one fourth of the coast for which strandings are reliably recorded is the English North Sea coast, yet the proportion of strandings for this coast is well below one quarter of the total for four of the eight decades represented. For the decades before 1970, when North Sea strandings were more frequent, Evans (1980) attempted to explain the rise by suggesting that it may have been due to elevated mortality arising from increased pollution and disturbance or over-exploitation of food sources in this part of the North Sea, rather than an actual increase of individuals in the area. Further support for generally low numbers on this coast comes from records kept in Denmark, which state that this species has never occurred at a very high frequency in the North Sea (Kinze 1995).

I.2 Flixborough

The state of bottlenose dolphins along the North Sea coast of England shown through strandings and sightings becomes even more interesting in the context of bottlenose dolphin population dynamics when the excavation of an Anglo-Saxon site near the modern village of Flixborough, England, is discussed. At this site, by far the largest archaeological find of bottlenose dolphin remains in Britain has been uncovered. The location of the settlement, referred to from here on as Flixborough, along the North Sea coast, is shown in Figure 1.3. The Anglo-Saxon site is located on a sand promontory into the floodplain of the River Trent, approximately 1.5 km from the river and 8 km south from the mouth of the Humber Estuary (Humber Archaeology Partnership/English

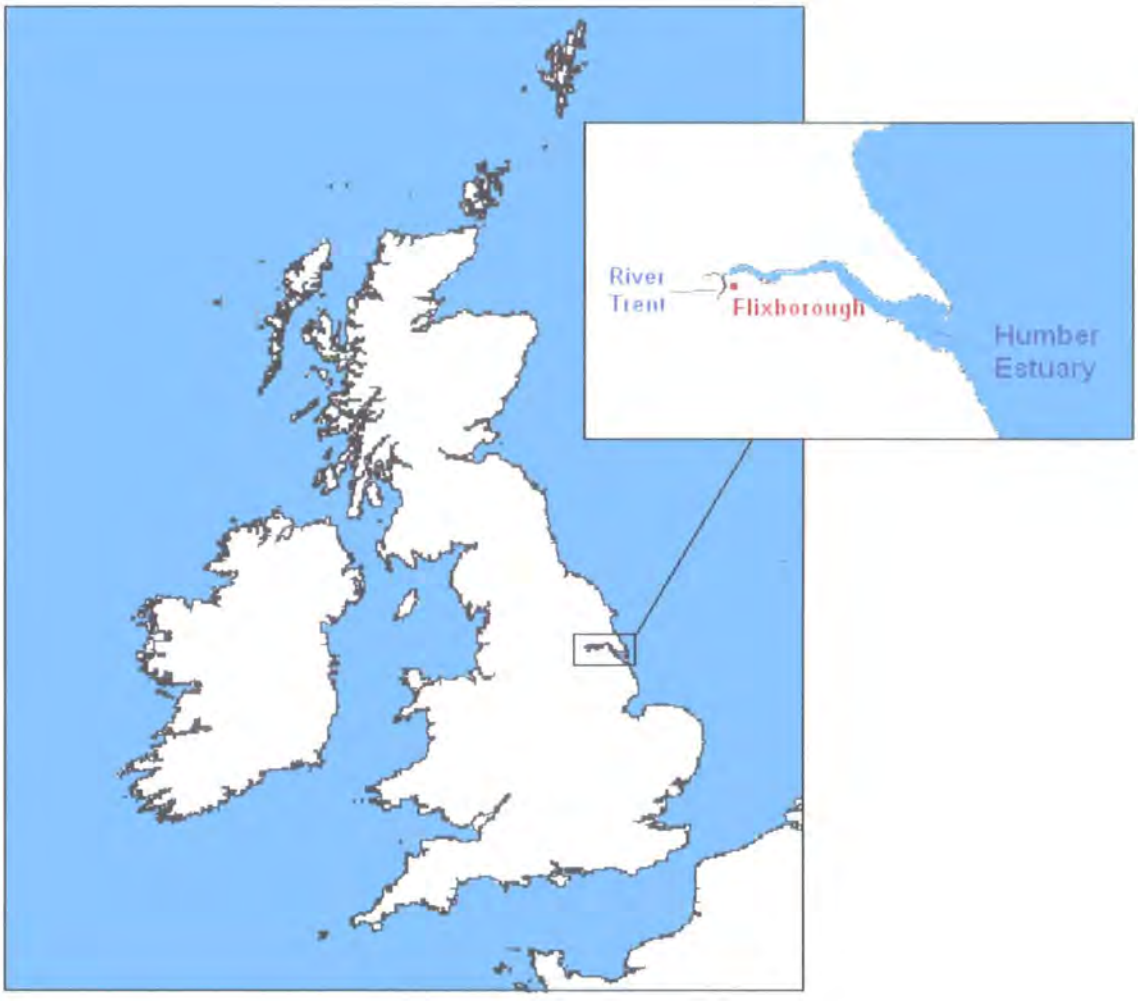

Figure 1.3. Map of the UK, with an inset of the Humber Estuary and its surrounding area. The River Trent branches off of the estuary, and the Flixborough settlement was located along the river.

Heritage, Loveluck 1997). Excavations have shown a very wealthy and high-status settlement that was inhabited by Anglo-Saxons from the 7th century into the early decades of the 10th century (Loveluck 1997).

Many artifacts and animal bones were recovered from the refuse dumps. These were preserved quite well despite the highly acidic sand geology of the site, as there was also a high alkaline wood ash content in the dumps that created neutral soil conditions (Humber Archaeology Partnership/English Heritage). Surprisingly, within the food remains, 154 cetacean bone fragments were found, which is an unusually high number for a British site of this era. They were found throughout all phases of the site and nearly all were identified to species as *Tursiops truncatus* (bottlenose dolphin) and then determined to represent a minimum of 58 individual animals (Herman and Dobney unpublished). Herman and Dobney (unpublished) concluded that only ten fragments are from other species, nine likely to be minke whale (*Balaenoptera acutorostrata*) and one possibly from a pilot (*Globicephala melas*) or killer whale (*Orcinus orca*). Nearly all of the fragments are pieces of skull, mandible, ribs, and dorsal and lumbar vertebrae. The type of bone fragments, which are all associated with large muscle blocks or reserves of oil, and butchery marks consistent with removal of meat and in one case oil-bearing tissue, suggest they were exploited for their food and oil and that only these useful parts of the animal were transported to the settlement from the coast (Herman and Dobney unpublished).

I.2.1 Archaeological Significance

This assemblage of bones is unique archaeologically because no excavation of a contemporary British site, to this date, has uncovered anywhere near this number of cetacean bone fragments, especially corresponding to such a large number of individuals. Gardiner (1997) reviewed reports of cetacean (whale, dolphin and porpoise) bones found at

Anglo-Saxon sites and noted that they are rare, coastally distributed, and have never consisted of more than 13 fragments, with the exception of a find in Hampshire of 60 fragments, which could have originated from only two whale carcasses.

The ceatacean assemblage at Flixborough is also of interest because it is made up almost entirely of fragments from bottlenose dolphins. Herman and Dobney (unpublished) studied this in the context of the cetacean stranding records for the British and Irish coasts kept by the British Museum of Natural History (Harmer 1927; Fraser 1934, 1946, 1953, 1974; Sheldrick 1989, 1994) and concluded that if the animals utilized at Flixborough originated from strandings, and if it is assumed that the fauna in the North Sea have not changed drastically in the last thousand years, the assemblage should at least include, and perhaps be dominated by, fragments from the harbor porpoise (*Phocoena phocoena*) and the white-beaked dolphin (*Lagenorhynchus albirostris*). Both of these species showed higher frequencies of strandings than the bottlenose dolphin over the last century, the harbor porpoise quite remarkably so (Harmer 1927; Fraser 1934, 1946, 1953, 1974; Sheldrick 1989, 1994). This comparison, along with the fact that most of the fragments are from adult and subadult individuals, when juveniles and especially calves are also likely to strand, led to Herman and Dobney's (unpublished) conclusion that Flixborough represents the first evidence for an indigenous cetacean fishery in the British Isles.

Gardiner (1997) reported that there is little evidence for cetacean fisheries in the British Isles, whether from archaeological finds or written records, until medieval times. Therefore, Flixborough may be quite important for its suggestion that cetacean fisheries were more widespread and of earlier existence than is suggested from other sources (Herman and Dobney unpublished).

I.2.2 Biological Significance

The collection of cetacean bones found at Flixborough is intriguing biologically because the site lies on England's North Sea coast, an area of coastline that bottlenose dolphins have not frequented for at least the last century. Yet, with the large number of animals represented by the fragments at Flixborough, it seems likely that there was a significant number, or perhaps even a resident population, of this species around the Humber Estuary during the 7th to 10th centuries.

This brings up several possible scenarios as to what changes have occurred in bottlenose dolphin populations over the last 1,000 to 1,300 years. Perhaps this is another example of the transient nature UK populations have shown in the last century and the population moved or contracted due to a change in environmental state or prey distribution. Alternatively, as Herman and Dobney (unpublished) suggested, the hunting of these animals, which appears to have intensified over the time of occupation according to rising numbers of fragments in successively later phases, may have led to the eventual extinction of the resident population in the estuary. Otherwise, these animals may have been imported to this high-status settlement from other areas of Britain, or even mainland Europe, signifying that there actually may not have been a population in the Humber Estuary during Anglo-Saxon times.

Determining which of these scenarios occurred would give valuable insight into population dynamics of bottlenose dolphins around the UK. As mentioned earlier, conservation of this species is a concern in British waters because there are only meager numbers that occur primarily in small, perhaps isolated, populations where there is fear of their decline (Evans 1980; Kayes 1985; Simmonds 1994). Parsons et al. (2002) and Thompson et al. (2000) noted the importance of using alternative approaches, in addition to field observational studies, to determine potential threats and the status of these animals.

This is especially important in the study of cetaceans because difficulties in field studies due to their life history traits, such as longevity, time spent under water, and ability to travel long distances, may mean that populations could decline to dangerously low levels before concern was raised. The more that is understood about the behavior and interactions with other populations and with their environment of past and present bottlenose dolphin populations in UK waters, the better current populations will be able to managed.

Herman and Dobney (unpublished) have used past literature to suggest a likely reason for this possible change in bottlenose dolphin population distributions since the 10th century, and this project uses the techniques of molecular genetics to further address this question. To accomplish this, variable genetic markers are studied in animals from the Flixborough site and compared with the same markers in modern animals from British waters and other populations throughout the world.

I.3 Selected Genetic Markers

I.3.1 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is a circular strand of duplex DNA that is located outside of the cell nucleus, within the cytoplasmic mitochondria. The molecule contains genes coding for a small number of proteins involved in electron transport, ribosomal RNAs, and transfer RNAs, along with a non-coding control region that is involved primarily in replication (Kasamatsu 1971; reviewed in Brown 1985). There is a lack of introns between transcribed genes in the molecule and intergenic sequences are either quite small or entirely absent (reviewed in Brown 1985; Moritz et al. 1987). Still, the rate of mutation in mtDNA is five to ten times faster than that of nuclear DNA (Brown et al.

1979; Brown et al. 1982), making mtDNA extremely useful for studies of genetic divergence between closely related species (e.g. Pichler et al. 2001; Wynen et al. 2001) and populations within a single species (e.g. Bickham et al. 1996; Parsons et al. 2002). The maternal inheritance of mtDNA (Hutchinson et al. 1974), as opposed to the biparental inheritance of nuclear DNA, means that it can be used in combination with nuclear markers to elucidate gender-specific gene flow (e.g. Hoelzel et al. 2001; Lyrholm et al. 1999; Palumbi and Baker 1994), but also means that the effective population size in all analyses is reduced to 25% of that in studies of nuclear DNA.

There are many mitochondria present within each cell, each containing its own copy of the mitochondrial genome, so there is a much greater abundance of mtDNA than nuclear DNA within each cell. This means that the molecule is preferable to work with when samples for genetic study have low DNA quantitites (Machugh et al. 2000). This is the case for most archaeological samples, such as the bones recovered from Flixborough.

Various regions of the mitochondrial genome have been used for genetic studies, but the highest resolution can be achieved through study of control region sequences. The majority of the high mutation rate of mtDNA stems from this region, as the level of sequence divergence is much higher than that of the coding regions of mtDNA (Greenberg et al. 1983). Within the control region, this great divergence occurs in a few spots of high variation among other areas of conserved sequences, which are likely needed for preservation of genetic function (Greenberg et al. 1983). Baker et al. (1993) and Bickham et al. (1996) have confirmed high levels of sequence variability in the mtDNA control region within populations of some marine mammal species, humpback whales (*Megaptera novaeangliae*) and Steller sea lions (*Eumetopias jubatus*), respectively. Additionally, Hoelzel et al. (1998), Parsons et al. (2002) and Wang et al (1999) found sufficient variability within this region for differentiation of bottlenose dolphin populations.

I.3.2 Microsatellites

Microsatellites are short segments of DNA characterized by a core sequence consisting of a repeated sequence motif ranging from two to six bases. They exhibit extreme variability between individuals in the numbers of the repeated sequence motif. This variation is thought to originate from strand slippage during DNA replication that can occur over the repetitive elements of the segment (Schlotterer and Tautz 1992). This exceptional variation makes these markers useful for resolving slight divergences between populations within a species that may not be possible to distinguish using less variable markers.

Microsatellites are found in large numbers spaced throughout the genome (Edwards et al. 1991), yet most have been demonstrated to be selectively neutral. Such high variation in a coding region of the genome likely would lead to a loss of function in a gene, which is perhaps the reason why these markers are generally found in non-coding, neutral regions of the genome. This selective neutrality is essential to their usefulness in population studies because it means they are compatible with the assumptions of population genetic theory.

Because of their high variability, selective neutrality, and relative ease to study, microsatellites have become commonly used genetic markers. They can be used to assess various characteristics of individuals and populations from paternity and kinship (Queller et al. 1993) to genetic variation within, between, or overall in species populations (e.g. Comstock et al. 2002; Garcia De Leon et al. 1997; Gotelli et al. 1994; Nyakaana and Arctander 1999; Paetkau and Strobeck 1994; Paetkau et al. 1999). Their usefulness in demonstrating population divisions in marine mammals, including bottlenose dolphins, has been exhibited in various studies (e.g. Berube et al. 1998; Hoelzel et al. 1998; Hoelzel et

al. 2001). The small size of many identified microsatellites also makes their use feasible for the study of material where degradation has reduced the length of most DNA molecules, and their successful use is documented in studies of archaeological and museum specimens (Kurosaki et al. 1993; Neilsen et al. 1999a; Wandeler et al. 2003; Zierdt et al. 1996).

I.4 Ancient DNA

The study of genetic markers in archaeological materials requires an entrance into the field that has been titled "Ancient DNA" or "aDNA". This is a relatively new field that strives to study the genetics of specimens whose exposure over time to environmental conditions has left samples with DNA of very low quantity and quality. Early attempts in this field brought reports of successful DNA extraction from fossilized plants (Golenberg et al. 1990) and dinosaur bones (Woodward et al. 1994). Since that time, however, skepticism has helped to put into place stringent criteria for the acceptance of sequences retrieved from ancient specimens that have brought into question the authenticity of these early claims.

I.4.1 DNA Degradation

The difficulties inherent in working with aDNA arise because DNA degrades over time. All DNA, in living organisms and in their remains, is subject to damage caused by hydrolysis and oxidation that can cause changes in the structure of the bases of DNA (reviewed in Lindahl 1993). In living cells, enzymatic processes exist to correct this damage, generally before they lead to actual heritable mutations in the genome (reviewed in Lindahl 1993). However, these mechanisms are no longer active once cells have died,

leading to the degradation of DNA molecules over time into short fragments that may have modified or missing nucleotide bases (Lindahl 1993; Paabo 1989).

The rate of DNA decay has been shown to be variable and dependent on the environmental conditions to which the molecule is exposed. It is generally accepted that preservation is best achieved at low temperatures (Lindahl 1993; Poinar and Stankiewicz 1999), neutral pH (Thomas and Paabo 1993), and in samples that were quickly dehydrated after death, which leads to lower rates of damage by hydrolysis (Lindahl 1993; Paabo 1989). Furthermore, although aDNA has been retrieved successfully from soft tissues, bone has been suggested to be a superior source of DNA because the binding of the molecule to hydroxyapatite, an inorganic component of bone, leads to a decrease in depurination rates (Lindahl 1993; Tuross 1994). The histological state of bone has also been shown to affect the preservation level of DNA (Barnes et al. 2000), perhaps because a stronger, denser covering provides greater protection from the environment and microbial infestation (Machugh et al. 2000). Teeth are also proposed to be good sources of aDNA for similar reasons (Ginther et al. 1992; Merriwether et al. 1994).

Despite the source, aDNA inevitably is present in low copy numbers and poor quality, and has been exposed to an environment where modern, high-quality DNA abounds. This reality leads to the three main challenges of aDNA work: to retrieve sufficient amounts of DNA from specimens, to accurately obtain sequences and genotypes from the molecules acquired, and to exclude modern DNA contamination throughout these processes.

I.4.2 Ancient DNA Challenges

I.4.2.1 DNA Retrieval

The successful retrieval of aDNA involves not only simply extracting the minute amounts of DNA present in these samples, but also keeping out of the extract the many impurities that often co-purify with DNA and are present in exceptional amounts in ancient specimens. This problem is addressed during extraction. Commonly used extraction procedures for aDNA include extra steps to concentrate the DNA obtained and wash away larger amounts of impurities. DNA is concentrated by catching the molecules in solution on Centricon™ microconcentrator membranes (Hagelberg and Clegg 1991), silica beads (Hoss and Paabo 1993), or silica-based columns (Yang et al. 1998). Impurities are then washed away with multiple wash steps before the DNA is eluted from these materials. The silica-based methods are superior at this junction because they specifically bind DNA, while the Centricon™ membranes will also retain any impurities that have a molecular weight above the cutoff of the membrane (Yang et al. 1998). Extra purification steps, such as isopropanol precipitation, have also been used to further purify aDNA (Hanni et al. 1995).

I.4.2.2 Accurate Sequencing and Genotyping

Retrieval of clean DNA, however, is only the first step in obtaining accurate sequences or genotypes from ancient sources. To visualize useful information about the molecule, many copies of the area of interest must be made. Early successes in aDNA used bacterial cloning to overcome this obstacle (Higuchi et al. 1984; Paabo 1985), but this procedure is complicated by the large amounts of damage that are typical in aDNA molecules. Damaged molecules result in low cloning efficiencies, and there is a danger

that damaged sites may be repaired through error-prone mechanisms within the bacteria, leading to a colony of incorrectly copied molecules (Paabo et al. 1989). The development and widespread use of the Polymerase Chain Reaction (PCR) has provided a superior alternative to cloning. In this amplification method, the majority of damaged molecules will not be duplicated because damaged sites interfere with the DNA polymerase, yet the reaction is sensitive enough to amplify segments from a very small number of intact molecules (Paabo et al. 1989). Furthermore, there is no capacity for repair within this *in vitro* reaction, meaning there also is no capacity for misrepair (Paabo et al. 1989).

Although PCR has advanced the field of aDNA greatly, the method still has its drawbacks. First, the requirement of intact, undamaged DNA segments for amplification means that only quite small fragments, generally less than 200 basepairs (bp), are amplifiable (Nielsen et al 1999b; Paabo 1989). Second, PCR may not always produce accurate sequences or genotypes from aDNA molecules. Hansen et al. (2001) reported that damaged sites on the molecule may become miscoding lesions during PCR and lead to the incorporation of an incorrect base. In addition, Paabo et al. (1990) found evidence that the polymerase used in PCR may jump to another template and continue polymerization when it reaches the end of a fragmented DNA molecule, thus creating an *in vitro* recombination product. It is argued that these instances of incorrect amplification are undetectable when direct sequencing is used to create a consensus sequence where the large majority of molecules were copied correctly (Paabo et al. 1989, 1990). Nevertheless, when genotyping for an allele size, such as microsatellite length, or when amplification was initiated on an exceptionally minute number (perhaps even one) of templates for sequencing or genotyping, these inaccurate products may be detectable (Paabo et al. 1990). In these cases, the published criteria for aDNA sequence verification must be relied upon to bring the discrepancy to notice.

PCR also may yield inaccurate results in cases where diploid nuclear DNA is investigated due to the phenomenon of allelic dropout. Allelic dropout occurs when only one of the two alleles present in a heterozygous sample is amplified to a detectable level. The phenomenon has been noted in many studies where the DNA source is of low quantity or quality, such as hair (Gangneux et al. 1997; Morin et al. 2001), faeces (Creel et al. 2003; Goossens et al. 2000; Morin et al. 2001; Parsons 2001), and ancient teeth and bones (Meyer et al. 2000; Ramos et al. 1995). Some of the criteria outlined later in this chapter for verifying aDNA sequences are also applicable for investigating the possibility of allelic dropout, and additionally, specific statistical criteria for acceptance of diploid genotypes from DNA sources of poor quality have been suggested in several publications (see Gagneux et al. 1997; Miller et al. 2002; Navidi et al. 1992; Taberlet et al. 1996).

I.4.2.3 Contamination

Even when sufficient copy numbers of fairly intact DNA survive to allow for successful PCR amplification, the danger of much larger copy numbers of high-quality contaminating DNA out-competing the endogenous DNA in the PCR reaction is present. Contamination of ancient specimens with modern DNA can come from microbes (during burial), from modern samples (during comparisons for identification) or from humans (throughout excavation, cleaning, identification, and storage). Furthermore, contamination of materials, solutions, or extracts can occur at anytime during aDNA laboratory work from modern DNA present on laboratory work surfaces, in the air, and on those performing the work. This constant threat of contamination has led to standard recommendations for avoiding contamination that are summarized by Machugh et al. (2000).

Ancient specimens should be cleaned thoroughly before extraction by methods such as soaking or wiping with a diluted bleach solution and removal of the outer layer of the specimen through use of a sand-blaster, sandpaper, or scalpel blade. Also, exposure of external surfaces to UV irradiation is useful for cross-linking, and therefore making unavailable for amplification, DNA molecules on the outer surface. Contamination during laboratory work can be avoided by wearing protective clothing, working in a positive pressure room or laminar flow hood, and cleaning all surfaces and materials with bleach and exposure to UV irradiation in between uses. Furthermore, sterilization of all materials and solutions is necessary before use, along with their dedicated use for aDNA work. And finally, pre- and post-PCR work areas should be physically separated and a one-way working procedure should be adhered to, where work on ancient specimens is never done after work with post-PCR stages.

Properties of the primers used in PCR can also aid in keeping any contaminating DNA from being represented in the results. Species specific primers can be designed that will not amplify sequences other than that of the species being studied. When working with animals, this can avoid the major contamination hazards of human and microbial DNA and give confidence that results obtained are from endogenous DNA (Richards et al. 1995). Nevertheless, contamination of the specimen with DNA from the same species during identification and storage, as well as carry-over contamination from modern to ancient labs, remain a concern.

Therefore, measures for detecting contamination also must be put into use. Extraction controls, where the extraction procedure is carried out without any sample material, may show evidence of contamination during the procedure and should be used during all extraction procedures. Additionally, negative PCR controls should be included in all amplifications to help recognize contamination originating at this step.

I.4.3 Verification

Despite the advanced techniques and precautions discussed above, aDNA work still may give inaccurate results. To avoid use and publication of such erroneous information, several criteria for acceptance of aDNA results have been suggested and are reviewed by Cooper and Poinar (2000). The most basic of these criteria is reproducibility. Results should be repeatable from at least two independent extracts of a specimen. Furthermore, when human remains are studied or when novel results arise, duplication by a separate laboratory is valuable to confirm authenticity. Molecular behavior also is an indicator of the reliability of results. For example, large amplification products above a few hundred basepairs should not be possible to obtain from ancient specimens, mtDNA results should be readily acquirable if nuclear DNA is successfully amplified, and failures in some samples of amplification and/or sequencing should be expected. Additionally, results should make sense in the context of the study and of the species phylogeny. The ability to obtain DNA from other faunal material at the site also can give confidence that site and storage conditions allowed for the survival of endogenous DNA.

Further tests to ensure that results are genuine include DNA quantitation. A minimum of 100 to 1,000 molecules for origination of amplification may be needed to confirm that damaged DNA molecules or polymerase errors during PCR did not contribute substantially to the amplification product (Handt et al. 1996). The use of cloning was promoted by Kolman and Tuross (2000) as a way to verify the presence of both endogenous and contaminating DNA, as each would be expected to be present in extracts. Finally, measurements of preservation levels of other biomolecules, such as proteins (Poinar and Stankiewicz 1999), sugars, and polyesters (Logan et al. 1993) may help to determine the likelihood of DNA survival in a specimen. Or, actual levels of DNA

damage can be investigated by assessing amounts of modified bases (Hoss et al. 1996). Each of these criteria and tests can increase confidence in the reliability of aDNA results, but only rarely are all of the suggested measures put to use in one study due to the high demands on time and finances.

I.5 Objectives

The overall goal of this study was to gain an understanding of the events that may have occurred during, or since, the time of Anglo-Saxon inhabitation of the Flixborough site that would explain the presence of such a large collection of bottlenose dolphin bone fragments on a region of the British coast that this species has not frequented for at least the last 100 years. In order to accomplish this, highly variable genetic markers were assessed in both the Flixborough specimens and present day animals. Both mtDNA (Pichler and Baker 2000) and microsatellites (Nielsen et al. 1999a) have been used previously to investigate temporal changes in genetic diversity and population structure, respectively, in studies where historic specimens were compared with modern animals.

Completion of this study required successful extraction of endogenous DNA from the Flixborough specimens, and amplification of both mtDNA and selected microsatellites from the extraction products. The success of studies for both types of these markers in the Flixborough population is assessed. Mitochondrial DNA amplification products were directly sequenced and the results were carefully edited, while microsatellite PCR products were assessed for allele size. The results of both markers were judged reliable only after consideration of the published criteria for acceptance of aDNA results. Finally, statistical analysis of the data was used to estimate genetic variability within each putative population and divergence between populations.

Results showing a close relationship between the Flixborough samples and a modern population would suggest a redistribution or range contraction of the group over the last millennium. Alternatively, demonstration of differentiation of the Flixborough animals from all other populations could indicate a local extinction of animals in or around the Humber Estuary. In this case, differentiation could have resulted from a founder event where allele frequencies were distorted by sampling effects, and continued isolation maintained this differentiation. If this were the case, low variation would be expected within the Flixborough samples, remaining from the low diversity created by just a few founders, and if we have included samples representing the founding population we would find that Flixborough shared most alleles with that group. However, differentiation of Flixborough from all groups could also indicate that samples from the source population for Flixborough were not included in this study. This could be simply because of a lack of sampling of the population, or could mean the source population is extinct, suggesting the possible shift of a new population into the area since that time. If either of these scenarios were the case, we would likely see novel alleles in Flixborough. Finally, if these animals were hunted in one, or several, other areas and imported to this site, we would expect to see close ties to another population or a mixture of genotypes from divergent populations.

Chapter II – Materials and Methods

II.1 Materials

II.1.1 Flixborough Samples

The location of the Flixborough site is shown in Figures 1.3 and 2.1. English Heritage funded the excavation of the Anglo-Saxon settlement located on a sand promonotory into the River Trent flood plain, just inland from the Humber Estuary. The bottlenose dolphin specimens from the Flixborough excavation are a collection of teeth and bone fragments, consisting largely of vertebrae, ribs, and skull fragments. Pictures of a selection of these fragments can be seen in Figure 2.2.

The fragments were first identified and grouped into a minimum number of potential animals by Jerry Herman of the National Museum of Scotland. Identification was done by direct comparison of the bone fragments to collections of known species at the museum (pers. comm. Jerry Herman). Some fragments could not be identified as being *T. truncatus* but were listed only as being from an unidentified cetacean. Ten samples also were identified as belonging to larger whale species. Grouping of the fragments into a minimum number of potential animals was accomplished by taking into consideration the phase and context of the excavation from which each fragment was collected, the estimated size of the animal from which the fragment came, and the type of bone, along with the maximum number of that type per animal (pers. comm. Keith Dobney and Jerry Herman).

The phases of an archaeological excavation refer to the horizontal layers of the site, which are dated due to their content. The lowest phase number refers to the oldest layer. A context is a defined area within a phase. Different context numbers could be assigned, for example, to different midden dumps or to areas with differing soil types. Table 2.1 lists the Flixborough fragments from which DNA extraction was attempted in this study,

Figure 2.1. Map of the British Isles showing the location of Flixborough, the Humber Estuary and the general areas of collection for modern *Tursiops truncatus* samples from around the islands. Each ellipse represents a generalized area of collection and the number inside, or just outside, the ellipse is the number of samples from that region (*n*).

A)

B)

Figure 2.2. Photographs showing a sample of the Flixborough cetacean specimens. A) A selection of teeth and two jawbone fragments. B) A selection of vertebrae. (Photographs are from the Humber Archaeology Partnership / English Heritage website report on the Flixborough settlement)

Table 2.1. Flixborough fragments from which DNA extraction was attempted. For each fragment, the phase and context of the excavation in which it was found is listed, as well as the age category, size, and bone identification assigned by Jerry Herman. (Table 2.1 is continued on the following two pages.)

Sample Number	Phase	Context	Age Category	Size	Bone Identification
53	2-3a	4487	Subadult	300	Left rib c.9 fragment
52a	2-3a	4621	Subadult	300	Lumbar vertebra c.15 fragment
52b	2-3a	4621	Subadult	300	Left rib c.10 fragment
23a	2-3a	4963	Subadult	310	Atlas/axis vertebra (fused) fragment
23b	2-3a	4963	Juvenile	250	Tooth
23c	2-3a	4963	Juvenile	250	Tooth
23d	2-3a	4963			Unidentified cetacean bone fragment
21b	2-3a	5314	Subadult	300	Right rib 12, proximal fragment
19b	2-3a	5369	Adult	300	Left parietal fragment
30	3b	3600	Subadult	300	Left scapula fragment
27	3b	4322	Subadult	300	Caudal vertebra c.5 fragment
50b	3b	4323	Juvenile	250	Caudal vertebra c.2 fragment
50d	3b	4323	Juvenile	250	Left scapula fragment
50e	3b	4323			Cranial fragment, probably frontal or parietal at suture
45c	3b	5617	Adult	300	Lumbar vert.c.5 fragment
45d	3b	5617	Subadult	300	Left rib 2 fragment
55	3b	5653	Subadult	300	Left rib c.10 fragment
10	3b	5983	Subadult	275	Posterior part rostrum (end toothrow)
41	3b	6028	Adult	320	Thoracic vertebra 1 fragment
38	3b	6136	Juvenile	250	Lumbar vertebra c.12 fragment
44a	3b	6235	Subadult	275	Rostrum fragment (mid toothrow)
44i	3b	6235	Adult	330	Lumbar vertebra c.14 fragment
44j	3b	6235	Subadult	275	Caudal vertebra c.2 fragment

Sample Number	Phase	Context	Age Category	Size	Bone Identification
44l	3b	6235	Adult	330	Right rib 3 proximal fragment
47	3b	6441	Juvenile	250	Left rib c.9 fragment
51	3b	7687	Subadult	300	Left rib c.10 fragment
46	3b	8200	Adult	330	Left rib 2 proximal fragment
54	4-5b	1662	Subadult	300	Left rib c.4 proximal fragment
58	4-5b	2720	Subadult/Adult	c.320	Sternal rib fragment
2a	4-5b	3758	Adult	320	Cervical vertebra c.5
2b	4-5b	3758	Juvenile	275	Thoracic vertebra c.10 fragment
2d	4-5b	3758	Subadult/Adult	310	Right rib c.9 fragment
5a	4-5b	5193	Subadult	275	Thoracic vertebra c.12 fragment
5b	4-5b	5193	Adult	320	Left rib c.9 medial fragment
20a	4-5b	5252	Adult	320	Right dentary fragment
20b	4-5b	5252			2 associated cranial fragments
12b	4-5b	5503			Unidentified bone fragment
12d	4-5b	5503	Subadult	300	Cranial fragment, maxilla, left side
12e	4-5b	5503	Subadult	300	Lumbar vertebra c.12 fragment
42	4-5b	5553	Subadult	300	Caudal vertebra c.1 fragment
4	4-5b	6885	Adult	330	Caudal vertebra c.6 fragment
6a	4-5b	12057	Adult	300	Anterior part rostrum, premaxilla
6d	4-5b	12057	Adult	300	Thoracic vertebra c.10 fragment
6e	4-5b	12057	Adult	300	Thoracic vertebra c.2 neural spine
40b	6	1708	Subadult	300	Sternum fragment, anterior right side
7	6	1831	Subadult	300	Lumbar vertebra c.10 fragment
11c	6	3610	Subadult	300	Lumbar vertebra c.5 fragment, with 2 associated epiphyses
11d	6	3610	Subadult	300	Left rib 1, distal fragment
1b	6	3891	Adult		8 teeth
1d	6	3891	Subadult		8 teeth
1e	6	3891	Subadult	275	Thoracic vertebra c.8 fragment

Sample Number	Phase	Context	Age Category	Size	Bone Identification
1h	6	3891	Adult	325	Lumbar vertebra c.9 fragment
1j	6	3891	Adult	300	Lumbar vertebra c.6. fragment
9b	6	3891	Subadult	300	Thoracic vertebra c.7 fragment
9d	6	3891	Adult	325	Lumbar vertebra c.15 fragment
8c	6	5871	Adult	325	Skull fragment, maxilla/frontal, right side
3	6	6498	Subadult	310	Lumbar vertebra c.10 fragment
37	6	10296	Juvenile	250	Caudal vertebra c.4 fragment
33	6iii	1269	Adult	320	Thoracic vertebra c.2 fragment
34	6iii	1283	Adult	320	Lumbar vertebra c.5 fragment
32	6iii	1457	Adult	320	Thoracic vertebra c.5 fragment
29a	6iii	1459	Subadult		Tooth
29b	6iii	1459	Subadult	300	Thoracic vertebra c.4 fragment
26	6iii	1740	Subadult	300	Maxilla fragment (mid toothrow)
16	6iii	3452	Subadult		Tooth
39	6iii	7054	Adult	320	Caudal vertebra c.7 fragment
17	?	1688	Subadult		Tooth

along with the information used in their grouping into potential animals. A full list of the cetacean fragments excavated at Flixborough and their pertinent information is found in Appendix A.

II.1.2 Modern Samples

General locations of collection for specimens from around the United Kingdom and Ireland and the sample size from each area are shown in Figure 2.1. Broad representations of locations and sample sizes of specimens from other regions of the world that were used in mtDNA and microsatellite analyses are illustrated in Figure 2.3. All samples from around the United Kingdom and Ireland were used in both mtDNA and microsatellite analyses, but elsewhere, only the populations geographically closest to the British Isles and the Flixborough site were included in the microsatellite studies. These populations are indicated in Figure 2.3.

II.1.3 Previously Published Data

Mitochondrial DNA sequences of animals from Chinese populations were taken from Genbank (NCBI) and were published in Wang et al. (1999). Mitochondrial DNA sequences for Western North Atlantic samples are from Hoelzel et al. (1998) and sequences from all other modern animals, with the exception of the British Isles, are from Natoli et al. (2004). Additionally, 15 of the mtDNA sequences for animals around the United Kingdom are from Natoli et al. (2004), and 20 from the United Kingdom and Ireland are from Parsons et al. (2002). Results for microsatellite locus D08 in 27 of the samples from around the British Isles and for all other populations outside the UK also are from Natoli et al. (2004).

Figure 2.3. World map displaying very general locations of sample collection for putative populations around the globe that were used in mtDNA and microsatellite analyses. The location of the United Kingdom (UK) is indicated in a lighter font, but more detailed areas of sample location for the region are shown in Figure 2.1. Population abbreviations are: SAA=South Africa *aduncus*, AT=Africa *truncatus*, BS=Black Sea, CHT=Chinese *truncatus*, CHA=Chinese *aduncus*, GM=Gulf of Mexico, WNAC=West North Atlantic Coastal, WNAP=West North Atlantic Pelagic, MS=Mediterranean Sea, GAL=Galicia, P+G=Portugal and Galicia. Population locations and sample sizes (n) used in mtDNA analyses are: SAA n=38, AT n=16, BS n=14, CHT n=17, CHA n=19, GM n=14, WNAC n=29, WNAP n=25, MS n=34, and GAL n=14. Population locations and sample sizes (n) used in microsatellite analyses are: WNAC n=27, WNAP n=27, MS n=30, and P+G n=29. Populations used in the microsatellite analyses are marked with a red asterisk (*).

II.2 Methods

II.2.1 Ancient DNA Contamination Precautions

Standard measures recommended to avoid contamination in aDNA work were used at all times when working with the Flixborough specimens. All aDNA work was done in laboratories separate from those used for modern DNA and post PCR work. Sampling of archaeological specimens to obtain material for aDNA extraction was carried out in a teaching laboratory of the university. This was a room where no PCR or post PCR work was performed. All further work involving ancient samples was completed in a dedicated aDNA laboratory located in a separate building from the modern DNA and sampling laboratory. Neither the teaching laboratory nor the aDNA laboratory was entered if work had been carried out on modern DNA or on PCR products earlier in the day. All materials brought into these rooms either had never been in the modern DNA lab or had been thoroughly wiped down with an approximately 10% dilution of bleach (sodium hypochlorite) at least twice before being taken inside.

Sampling of the Flixborough specimens was done in a fume cupboard with the airflow switched off, and all work in the aDNA lab was completed in a laminar flow hood. All surfaces in the hoods were wiped down with a 10% dilution of bleach each time before work was begun and again after the work was completed. All materials and equipment used were dedicated for use on aDNA and were cleaned with diluted bleach before and after use and in between use on different samples. Drill bits used for sampling were soaked in diluted bleach for several minutes after each drilling session and were occasionally autoclaved. Water and solutions prepared for aDNA work were autoclaved and filtered through a 0.2 micron syringe filter. Pipette tips came certified sterile from the

manufacturer in individually wrapped packages, which were always unwrapped inside the aDNA lab and only opened inside the hood.

A laboratory coat dedicated for use with aDNA was used at all times, and plastic gloves were worn, which were taped to the lab coat sleeves to avoid exposure of wrist skin. Sleeves were worn over the lab coat and were wiped with 10% bleach preceding work. A hood to cover the head and contain hair also was worn while working in the aDNA lab.

Additional steps to avoid contamination were taken during DNA isolation and PCR amplification. Many are specific to each of those procedures, and therefore are discussed within their respective sections of the methods. Furthermore, controls were carried out in parallel with all isolations and PCR reactions to monitor for contamination. Details of the controls used are included in the discussions of the methods for ancient DNA isolation and PCR amplification. Procedures used to monitor authenticity in the results produced by mtDNA sequencing and microsatellite analysis also are addressed in their respective discussions of methods.

II.2.2 DNA Isolation

II.2.2.1 Ancient DNA

II.2.2.1.1 Sampling and Digestion

Ancient DNA obtained from the Flixborough specimens was extracted from bones and teeth. All samples in the collection that appeared to be structurally well preserved and were large enough to accommodate multiple extractions were selected for extraction.

Sampling of the specimens was accomplished using a drilling technique. Teeth were drilled through the natural cavity in the proximal end, into the area where the dental pulp had once been located. Two factors where taken into consideration when choosing

the surface of a bone to drill. Most importantly, the area of the bone that appeared most dense was chosen, as the densest areas of bone carry the most intact DNA since it has been more protected from the environment (Machugh et al. 2000). Secondarily, drilling was done on the most inconspicuous area of the bone possible. The Flixborough samples were given to a museum for display after analysis, and therefore, it was requested that sampling be as non-destructive as possible and that holes be made in areas that could be hidden during display.

The drilling procedure used was a slightly modified form of that used by Ana Topf (pers. comm.). In preparation for drilling, the samples were treated to remove any contaminating DNA that may have collected on the outer surfaces during the excavation and identification processes. Each sample was wiped down with 10% bleach and the top layer of the surface of a bone to be drilled was removed using fine grade sandpaper. The inner cavity of teeth was rinsed with 10% bleach. The specimen then was exposed to UV light at a distance ranging from 3 to 10 cm for a minimum of 15 minutes. The sample was rotated occasionally during this time to expose all surfaces to the irradiation. Finally, all surfaces of the specimen were wiped down again with diluted bleach before drilling commenced.

Drilling was done with a small, hand-held drill and Dremmel™ drill bits. Dremmel™ bits, designed for engraving use, have small, round or cylindrical knobs at their tips, which are covered with ridges. The bits were used to drill a small hole into the specimen and create a fine powder of the displaced material. The powder was collected into 10 mL tubes. The first powder created by the drilling of the outermost layer was discarded to avoid including any possible contamination of the specimen surface in the extraction sample. The design of the drill bits allowed a fairly large cavity to be created

inside most specimens, from which powder was collected, while only a small outer hole was needed. It was attempted to fill the collection tube up to the 0.5 mL point with powder; however, some specimens could not yield this much powder and extraction was carried out on less, and sometimes, very minute amounts of material (down to approximately 0.1 mL). Between the drilling of each sample, the work area, all materials, and gloved hands were wiped down with 10% bleach. At least one extraction control was begun at this step, by capping an empty tube and setting it aside in the fume cupboard at the start of drilling. All work following the drilling took place in the dedicated aDNA laboratory.

Enzymatic digestion of the samples was completed by adding between 3 and 8 mL of a high ethylenediaminetetraacetic acid (EDTA) digestion buffer (0.425 M EDTA, 0.5% sodium dodecyl sulfate, 0.05 M Tris) (Montiel pers. comm.), depending upon the amount of sample, which was judged by markings on the 10 mL tubes holding the powdered bone. Bone samples that produced only a small volume of powder, up to approximately 0.2 mL, received 3 mL of digestion buffer. Samples that yielded over 0.5 mL of powder were digested in 8 mL of buffer, and a gradient was used for all intermediate volumes. Individual solutions used to make the digestion buffer were certified sterile by their manufacturer or autoclaved and filtered if produced in the laboratory. After mixing of the digestion buffer, the total solution was exposed to UV irradiation for 10 minutes while in a sealed container, to destroy any potential DNA contamination that occurred during the preparation. Proteinase K was then added. Initially, it was used at a concentration of 0.133 mg/mL (suggested by Matisoo-Smith et al. 1997; Yang et al. 1998), but later in the project the concentration was increased to 0.333 mg/mL. Extraction controls were continued at this step by placing 2 to 3 mL of the digestion buffer, after it had been added to all samples, and proteinase K into the tubes that had been set to the side during drilling.

From this point forward, extraction controls were treated identically to all other sample digests. All digests were incubated on a rotating wheel at 55°C overnight and then at 37°C for 24 hours.

II.2.2.1.2 DNA Extraction

Initial titrations of the aDNA extraction procedure were carried out on modern bottlenose dolphin bones and teeth. Trials of two procedures for the actual DNA extraction and purification, following digestion, were completed on the modern samples and on the same digestion samples of three Flixborough specimens. These included a procedure composed by Ana Topf (pers. comm.). This protocol consisted of a standard phenol-chloroform extraction (Sambrook et al. 1989), after which, the resulting DNA in aqueous solution was bound to Qiaex II™ silica beads. The beads were then loaded onto a filter column where they were washed, and the DNA was eluted in 50 µL 1X TE (Tris-EDTA) buffer. The second method used was a modified form of the QIAquick PCR Purification Kit™ method recommended in Yang et al. (1998). This method was used in all further aDNA extractions and is described in greater detail below.

In the modified form of the QIAquick™ method, 1.4 mL of the sample digest was placed in a capped tube and centrifuged at 5,100*g* for 5 minutes to separate out solid materials remaining after digestion. The supernatant was transferred to a clean tube and spun again at 12,800*g* for 5 minutes. The supernatant was then added to a 5x volume of QIAquick™ PB Buffer and the tube was slowly inverted several times for mixing. A 700µL portion of the solution was then loaded directly onto a QIAquick™ column and centrifuged at 12,800*g* for 1 minute. The flowthrough was discarded and the previous step was repeated until the entire solution had been passed through the column. The DNA was

then washed by adding 700 µL of QIAquick™ PE Buffer to the column, centrifuging for 1 minute at 12,800*g*, and discarding the flowthrough. The above washing was repeated and the column was spun for an additional 1 minute to ensure removal of all traces of PE Buffer. The DNA was eluted from the column by loading 50 µL of 1X TE buffer directly onto the column membrane, allowing this to sit for 10 minutes, and centrifuging for 1 min at 12,800*g*. The DNA solution was stored at -20°C.

II.2.2.2 Modern DNA

DNA from modern samples was isolated from pieces of skin that had been stored in a 20% DMSO / 5 M NaCl solution (Amos and Hoelzel 1991). A small sample, approximately a 3 mm cube, was cut from the specimen and finely chopped. The sample was digested at 37°C overnight in 500µL of digestion buffer (50 mM Tris pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% w/v SDS) (Milligan, B.G. 1998) with 0.6 mg/mL proteinase K. Total DNA was then extracted using standard phenol/chloroform extraction followed by ethanol precipitations using sodium acetate and then lithium chloride as the monovalent cations (Sambrook et al. 1989). DNA was stored in 1X TE buffer at an approximate concentration of 100-200 ng/µL at -20°C.

II.2.3 PCR Amplification

II.2.3.1 Mitochondrial DNA

A portion of the mitochondrial control region d-loop was amplified for the study of mtDNA sequence variation. For the Flixborough samples, primers specifically for *T. truncatus*, but which in the end would also amplify mtDNA in some other odontocete species, were designed. A 240 bp PCR product was produced and primer sequences were

5'-TTAGTCTCTCCTTGTAAAT and 5'-GGTGATTAAGCTCGTGAT. For each sample 5 μL of the 50 μL DNA extract was used (the amount of DNA in aDNA extracts was not quantified) in a 25 μL PCR reaction consisting of 15 mM Tris-HCl pH 8.0, 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 0.08 μg/μL bovine serum albumin (BSA), 26 ng/μL of each primer, and 0.5 units PE Applied Biosystems AmpliTaq Gold™. It is important in aDNA work that the Taq polymerase be a hot start enzyme. The sealed reaction tubes were then taken as quickly as possible to a PCR cycler in the modern DNA lab. Cycle conditions were 8 minutes at 95°C, 46 cycles of 45 seconds at 94°C, 1 minute 30 seconds at 57°C, and 1 minute 30 seconds at 72°, followed by a final extension step of 10 minutes at 72°C. Samples were stored at 4°C. Alongside amplification of all samples, PCR amplification was also attempted on all extraction controls to check for contamination that may have occurred during the extraction. A negative PCR control, using 5 μL of water instead of a DNA extract, was also included to test for contamination that took place during the set-up of the PCR reaction. Finally, a positive control, using 4 μL of water and 1 μL of a 100-200 ng/μL solution of modern bottlenose dolphin DNA, was included to ensure the PCR reaction was working. The DNA for the positive control was added to that reaction in the modern lab after all other samples had been placed in the PCR cycler and just before starting the machine.

In modern samples, an approximately 1000bp region of the mtDNA control region was amplified. Universal primers for mammalian mtDNA of sequences 5'-TTCCCCGGTCTTGTAAACC and 5'-ATTTTCAGTGTCTTGCTTT were used (Hoelzel and Green 1998). Reaction conditions were 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 1.0 mM $MgCl_2$, 10 ng/μL of each primer, and 0.6 units of Invitrogen™ Platinum Taq polymerase. Approximately 50 to 100 ng of DNA was used in each

reaction; however, when samples were difficult to amplify, a 1:10 dilution of DNA was used in an effort to lower possible levels of PCR inhibitors in the solution. Cycle conditions were 8 minutes at 95°C, 36 cycles of 1 minute at 50°C, 1 minute at 72°C and 1 minute at 95°C, followed by an extra 1 minute at 50°C and a final extension of 8 minutes at 72°C. Samples were stored at 4°C.

II.2.3.2 Microsatellite Loci

Five microsatellite loci were used in this study. They were chosen because of the small sizes of their amplification products, which made them more likely to amplify in aDNA. These loci and their primer sequences are listed in Table 2.2 along with the $MgCl_2$ concentration and annealing temperatures used for amplification in both ancient and modern samples. To allow sizing of the PCR product using ABI Prism™ technology, one tenth of one of the primers in each reaction was from a primer solution in which the oligonucleotides had been labeled at the 5' end with a fluorescent ABI Prism™ dye. The primer that was labeled in each set is noted in Table 2.2.

In the Flixborough samples, PCR amplification of the microsatellites was carried out in 25 µL reactions using 5 µL of DNA extract. Later in the study, some reactions were carried out using 3 µL of the DNA solution in an effort to conserve volumes of the extracts. However, poor results led to a return to the use of 5 µL. Reaction conditions were 15 mM Tris-HCl pH 8.0, 50 mM KCl, 0.2 mM each dNTP, $MgCl_2$ at the concentration specified in Table 2.2, 0.08 µg/µL BSA, 26 ng/µL of each primer and 0.5 units PE Applied Biosystems AmpliTaq Gold™. Cycle conditions were 8 minutes at 95°C, 46 cycles of 45 seconds at 94°C, 1 minute 30 seconds at the annealing temperature (see Table 2.2), and 1 minute 30 seconds at 72°, with a final extension step of 10 minutes at

Table 2.2. Microsatellite loci, their primer sequences, and the PCR conditions used with ancient and modern samples. The primer sequence marked with an asterisk was flourescently labeled.

			Ancient Conditions		Modern Conditions	
Locus	**Primer Sequences**	**Source**	**$MgCl_2$ Conc.**	**Annealing Temp.**	**$MgCl_2$ Conc.**	**Annealing Temp.**
Ttru AAT_{44}	5'-*CCTGCTCTTCATCCCTCACTAA 5'-CGAAGCACCAAACAAGTCATAGA	*Caldwell et al. 2002*	1.5 mM	52°C	1.5 mM	55°C
D22	5'-*GGAAATGCTCTGAGAAGGTC 5'-CCAGAGCACCTATGTGGAC	*Shinohara et al. 1997*	1.3 mM	56°C	1.5 mM	59°C
D08	5'-*GATCCATCATATTGTCAAGTT 5'-TCCTGGGTGATGAGTCTTC	*Shinohara et al. 1997*	1.5 mM	59°C	1.5 mM	57°C
MK8	5'-*TCCTGGAGCATCTTATAGTGGC 5'-CTCTTTGACATGCCCTCACC	*Krutzen et al. 2001*	1.3 mM	62°C	1.3 mM	62°C
D18	5'-*CCCAAAACCGACAGACAGAC 5'-GATCTGGGGATGCAGG	*Shinohara et al. 1997*	1.8 mM	62°C	1.0 mM	60°C

72°C. Samples were stored at 4°C. The PCR machine used for cycling was located in the modern DNA lab, as explained for mtDNA amplification, and negative and positive PCR controls were also included as described in the section on mtDNA amplification.

In modern samples, microsatellite amplification was carried out using approximately 50 to 100 ng of DNA, although as with the mtDNA amplifications, 1:10 dilutions of this were used in some difficult samples. For all loci except MK8, reaction conditions were 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton® X-100, 0.2 mM of each dNTP, $MgCl_2$ to the concentration specified in Table 2.2, 10 ng/μL each primer and 0.3 units of Taq polymerase. For locus MK8, the same reaction conditions used for the Flixborough samples were used with modern samples, with the exception of the absence of BSA and the use of only 10 ng/μL of each primer. This was due to difficulties in titration with a polymerase other than PE Applied Biosystems AmpliTaq Gold™. Cycle conditions were 5 minutes at 95°C, followed by 36 cycles of 45 seconds at 94°C, 1 minute 30 seconds at the annealing temperature (see Table 2.2), and 1 minute 30 seconds at 72°C, plus a final extension step of 10 minutes at 72°C. Samples were stored at 4°C.

When difficult samples in all loci could not be amplified using the above conditions and a 1:10 dilution of DNA, they were amplified using the aDNA reaction conditions for the loci, but with only 10 ng/μL each primer and 36 cycles in the amplification cycle. The hot start Taq polymerase and the BSA, which binds inhibitors, often aided in allowing a successful reaction.

II.2.4 Gel Electrophoresis

Products of modern DNA extractions and all PCR amplification reactions were visualized using agarose gel electrophoresis. Gels were made at a 1% concentration of

agarose in 1/2X TBE buffer (44.5 mM Tris-borate and 1 mM EDTA at pH 8.3) and included approximately 0.5 μg/mL of ethidium bromide. Gels were ran at 100 volts for 20 to 30 minutes or until bands were sufficiently separated for clear interpretation. For all modern DNA extractions, 5 uL of extract was run for visualization on a gel. Also, 5 μL of all aDNA PCR products, were run on a gel. The same amount was also run for all modern mtDNA products, but only 3 μL of all modern DNA microsatellite products were run. DNA bands were visualized using UV light, and images were captured using Quantity One Gel Doc™ software.

II.2.5 Mitochondrial DNA Sequencing

II.2.5.1 Laboratory Methods

The remaining product of all successful mtDNA PCR amplifications, minus the 5 μL ran on the agarose gel, was purified using a Qiagen QIAquick PCR Purification Kit™ to remove primer dimers, unincorporated dNTPs, and unwanted chemicals. Occassionally, non-specific amplification products were obtained in addition to the expected product. In these cases, the Qiagen Gel Extraction Kit™ was used to purify only the desired PCR product. The instructions provided with the kits were followed. Purified aDNA products were eluted in 30μL, to concentrate the minute amounts of DNA, while modern DNA products were eluted in 50μL. The elution buffer was also allowed to sit on the column membrane for 5 minutes for aDNA products. The purified DNA was then sequenced by DBS Genomics (University of Durham) using the ABI BigDye Terminator sequencing kit™ and running of the products on an ABI Prism 373 or 377 polyacrylamide slab gel automated sequencer.

II.2.5.2 Interpretation of Results

Results of the sequencing gels were viewed and proofread using chromatograms produced by the automated sequencer. An example of a sequence chromatogram can be seen in Figure 2.4. Paper copies of the chromatograms were supplied, as well as computer files of the trace data from the sequencer. The trace files were viewed by importation into Sequencher 4.0 (Gene Codes Corporation). This software allows visualization of the sequence chromatograms, as well as the sequence text, and automatically aligns multiple sequences upon request. All sequences were trimmed to a 171 bp consensus sequence available for the aDNA samples. Sequence texts were proofread using the chromatograms, and peak heights could be adjusted using Sequencher 4.0 (Gene Codes Coporation) to better visualize peaks and allow clarification of difficult base calls. Furthermore, alignment of many sequences allowed comparison of peak structure and polymorphic sites, which also aided in clearing up basecalls.

Samples that showed ambiguous base calls were repeated to clarify; often with the primer not previously used. This was helpful as structures in the molecule that made the sequence difficult to determine when sequencing one strand would be absent at that location in the complimentary strand of DNA. The ends of sequences opposite from the primer utilized in the sequencing reaction were not used because they were generally unclear and difficult to read. The sequencing of each sample with both oligonucleotides of the primer set was used to obtain clear sequences for each end of the fragment. Primer sequences were not included in the sequence used for analysis. All aDNA sequences were duplicated a minimum of two times from different extractions before the results were accepted for analysis.

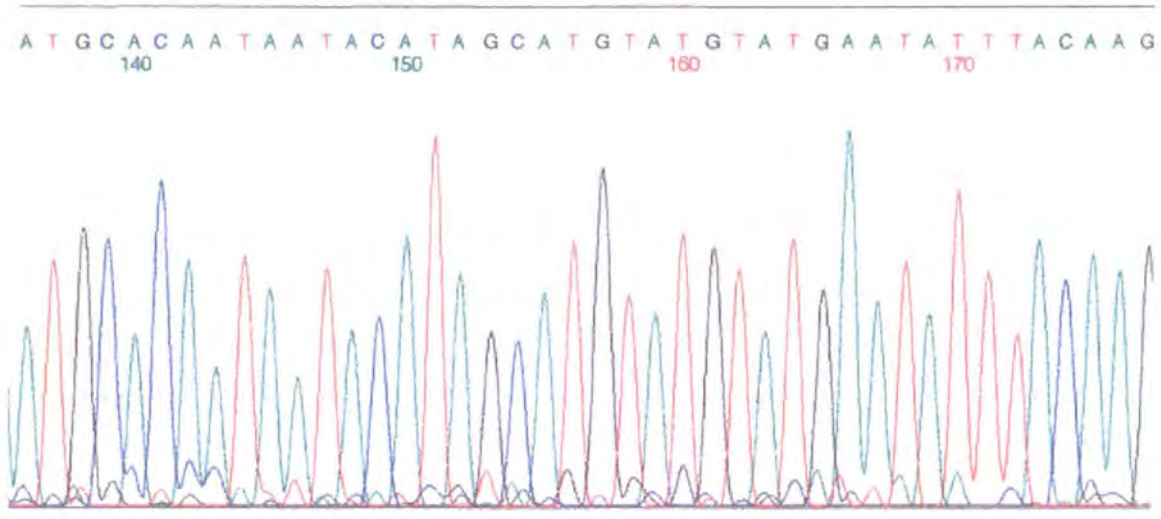

Figure 2.4. Portion of a sequence chromatogram resulting from automatic sequencing. Each peak signals a single nucleotide base, and each of the bases is represented by a color (Adenine=green, Cytosine=blue, Guanine=black, Thymine=red). The sequence is represented in text at the top of the figure (A=Adenine, C=Cytosine, G=Guanine, T=Thymine). The numbers under the text indicate the number of the base sequentially within the chromatogram.

II.2.6 Microsatellite DNA

II.2.6.1 Laboratory Methods

Microsatellite PCR products were run, without further purification, on polyacrylamide gels. They were run on the same 373 or 377 ABI polyacrylamide slab gel automated sequencers that were used for DNA sequencing, and this was also done by DBS Genomics. As mentioned in the earlier section on PCR, each product had been labeled by the use of a flourescently labeled primer, allowing the product to be detected by the sequencer. ABI Prism™ fluorescent labels of FAM, HEX, and NED were used. Running of a ROX labeled DNA size ladder in each lane allowed sizing of the detected PCR products.

II.2.6.2 Interpretation of Results

Visualization of PCR product sizes to a resolution of 1 bp was possible on a chromatogram produced by analysis of the output of the automated sequencer using ABI Genescan™ and Genotyper™ software. An example of a Gentotyper™ chromatogram used for sizing microsatellite products is shown in Figure 2.5.

Notable in Figure 2.5 are the 'stutter peaks' produced. 'Stutter peaks' refers to peaks produced by PCR products one or a few repeats smaller or larger than the actual microsatellite allele in the sample. Generally smaller stutter peaks are more prominent and persist further from the actual allele. These are created in the PCR amplification process by slippage of the polymerase during replication, in much the same fashion that the high variability of microsatellites is created in nature. Stutter peaks are most frequent and of highest amplitude in dinucleotide loci but are also found in repeats of higher nucleotide number. The peak of highest amplitude was designated as the allele size. However, stutter

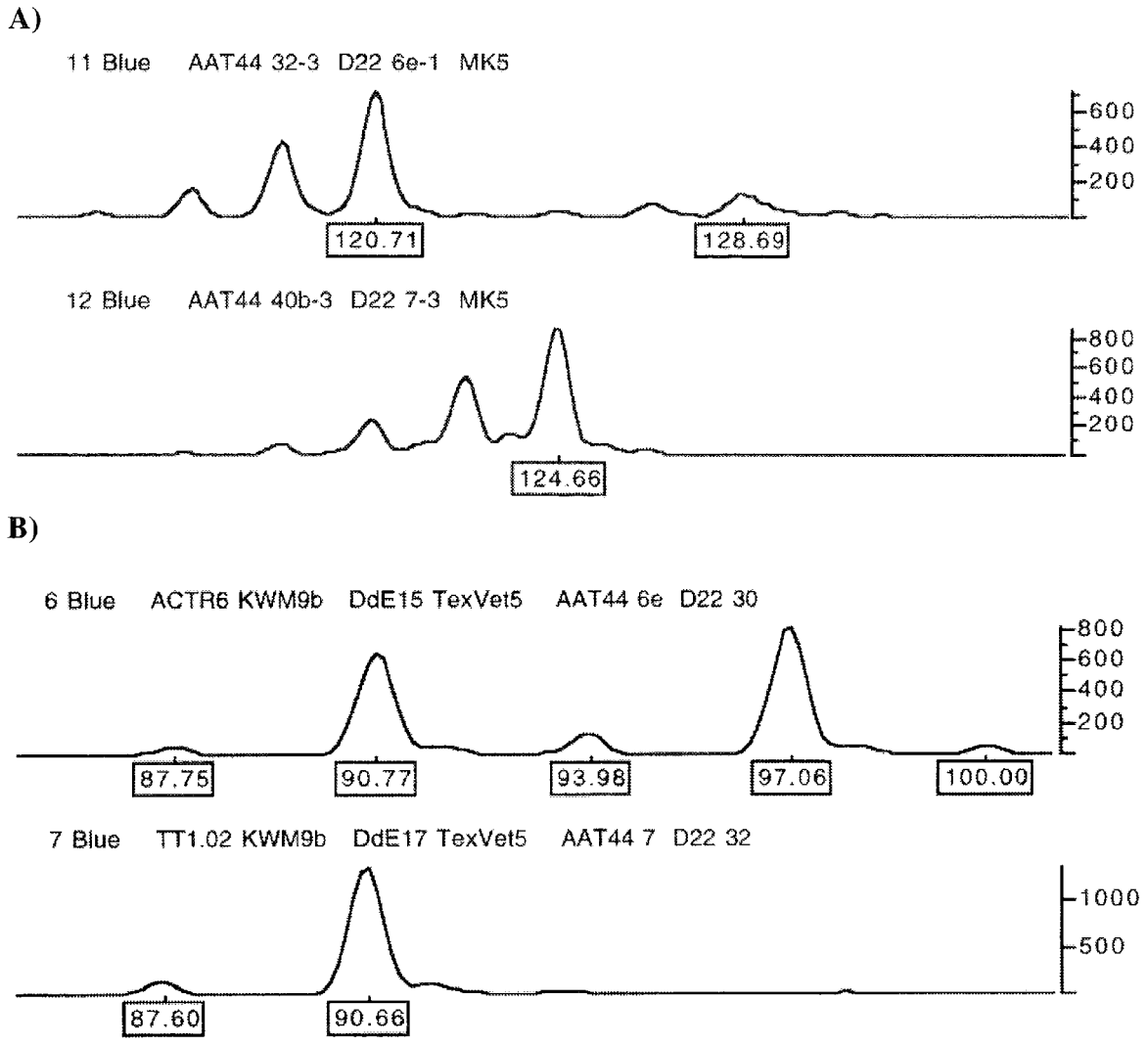

Figure 2.5. Examples of chromatograms shown by ABI Prism Genotyper™. The size of the DNA fragment, or PCR product, is shown in the boxes below the peaks. The numbers on the right margin are a scale for the peak amplitude. Information at the top of each lane shows the lane number and color of dye represented, followed by the samples in each lane. A) Two lanes showing dinucleotide repeat microsatellite structures. The top lane is a heterozygote (121 bp and 129 bp PCR products) and the lower is a homozygote (125 bp product only). B) Two lanes showing trinucleotide repeat microsatellite structures. Again the top lane is a heterozygote (91 bp and 97 bp products) and the bottom shows a homozygote (91 bp product only). Note the stutter peaks located above and below the high amplitude peaks that represent alleles. Each stutter peak is one repeat smaller or larger than the allele.

peaks were helpful in that they created a signature structure for alleles of each locus and were used in determining the validity of peaks appearing on the chromatograms.

Microsatellite alleles were considered reliable and used in the analysis if the peaks met certain criteria. First, the highest amplitude peak, used as the allele size, was only considered valid if it had an amplitude higher than 50 on the chromatogram. Most alleles, especially in modern samples, were well above this amplitude, and any peaks below 100 were duplicated before use in the analysis. Second, alleles deemed reliable had to show the expected signature structure. Each locus showed a pattern in the shape and prominence of the stutter peaks associated with an allele, and any peaks not showing this pattern were considered to be background 'noise' in the chromatogram or unspecific amplification. Finally, an allele was counted if it made sense in the context of the locus and the putative population of the individual. Only the area of the chromatogram around the expected range of each locus was screened for allele peaks, and alleles shown to be unique to a locus or to a putative population were only accepted after duplication.

II.2.6.3 Considerations for Ancient DNA Results

Rarely, in aDNA samples, the closest smaller stutter peak of an allele would have a slightly higher or equal amplitude to that of the actual allele, giving a false allele. An example of this phenomenon in an aDNA amplification can be seen in Figure 2.6. The occurrence of this in samples containing very small amounts of DNA is noted in Taberlet et al. (1996) and is attributed to slippage in the first rounds of the PCR amplification process.

Taberlet et al. (1996) set out three conditions for accepting this phenomenon. First, the intensity of stutter peaks shown in a locus should be proportional to the observance of these false alleles in the locus. Second, the most commonly observed of these false alleles

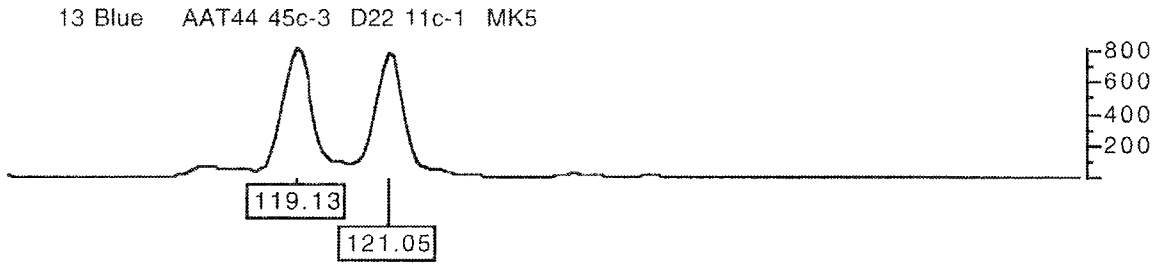

Figure 2.6. Example of a Genotypter™ chromatogram showing two adjacent peaks of nearly equal amplitudes, one possibly being a false allele due to slippage of the polymerase in early rounds of the PCR reaction. Note that there is only one, quite short, stutter peak of a length below 119 bp, suggesting that the peak at 119 bp does not represent an independent allele.

should match the most intensive stutter peaks. Finally, all intermediate peaks between the strongest and weakest signal stutter peaks must be present. When an allele was verified by multiple PCR amplifications, including those done on different extractions, and the above conditions were met, alleles in aDNA that had shown this phenomenon in one of their reactions were accepted for analysis.

As discussed in the introduction, microsatellites amplified in aDNA show an extremely high incidence of allelic dropout and false alleles due to the low quality of DNA and low copy number of nuclear genes preserved in archaeological samples. For this reason great care was taken in deciding when to accept an allele as reliable for use in analysis. The following guidelines, based on a "multiple-tubes approach" first put forth by Navidi et al. (1992), were adhered to in order to ensure accuracy of the genotypes. No allele was accepted as valid unless it had been reproduced in two PCR reactions from independent extractions of a sample. Acceptance of a heterozygous genotype was fairly straightforward if both alleles had been duplicated and no other peaks of a reliable structure or height had been observed. If a third allele had been observed among PCR repetitions of the sample, many duplications were needed to clarify the correct genotype. In only one case in this study was a heterozygote genotype accepted where a third reliable allele had been observed. The allele deemed to be false was seen in one repetition, and twelve other amplifications failed to show it again.

Homozygous genotypes are more difficult to accept as repeated allelic dropout of one allele could lead to a false homozygous genotype. Therefore, as many duplications as possible with the limited amount of DNA extract available were completed with all samples showing a homozygous genotype for a locus. Then, three data sets were accrued with varying criteria for acceptance of a homozygote. One data set accepted homozygotes that had been repeated only twice. Another set used only those homozygotes that had

been duplicated a minimum of five times. This acceptance level was based on reasoning put forth in Taberlet et al. (1996) that the probability of obtaining only the same single allele from a heterozygous individual is $0.5n$, where n is the number of replicate genotypings. Therefore, when $n = 5$ the probability of obtaining a false homozygote should be only 0.0313, less than 5%. The third data set used a criteria set forth in Gagneux et al. (1997) where the probability of allelic dropout for a locus was used to calculate the number of replications needed to reach a certain confidence level. The equation:

$$P(\textit{false homozygote}) = (K)((K/2)^{n-1})$$

where K is the observed frequency of false homozygotes average over all individuals and n is the number of repeated replications was used. Gagneux et al. (1997) did not calculate this probability individually for each locus; however, due to high variability in observed numbers of false homozygotes among loci used in this study, it was calculated separately for each locus here. The number of replications needed for acceptance of a homozygote in each locus was then determined as that number which allowed a *P(false homozygote)* of less than 0.05.

Initial analyses were run with each of the three data sets in order to determine how the level of homozygote acceptance for the Flixborough sample affected measures of intrapopulation variation and estimates of Flixborough's differentiation from other populations. Allele number and allelic richness, as calculated in FSTAT 2.9.3.2 (Goudet 2001), were used to evaluate levels of intrapopulation variability and F_{ST}, also as figured by FSTAT 2.9.3.2 (Goudet 2001), was used to investigate variations in population differentiation among the data sets.

II.2.7 Data Analysis

II.2.7.1 Determination of Putative Populations

The separation of samples into putative populations for use in analysis of both the mtDNA and microsatellite data was accomplished by considering geographic separation of samples, previously published accounts of genetic differentiation, and preliminary analyses completed with several different population divisions. These analyses were completed on the microsatellite data, as the higher variability of these markers makes them more likely to show fine population differentiation. Deviation from Hardy-Weinberg equilibrium was studied for each trial putative population, especially when possibly differentiated groups were pooled, to look for evidence of Wahlund's effect. Deviation from Hardy-Weinberg equilibrium was tested using an analog of Fisher's exact test with a Markov-chain method (100,000 iterations, 5,000 dememorization steps, sequential Bonferroni correction applied) as described in Guo and Thompson (1992) and calculated by ARLEQUIN 2.0 (Schneider et al. 2000). Pairwise population F_{ST} values using the methods of Weir and Cockerham (1984), as implemented by FSTAT 2.9.3.2 (Goudet 2001), were used to investigate levels of differentiation between proposed population groups.

Structure 2.0 (Falush et al. 2003; Pritchard et al. 2000), which uses a Bayesian based model to infer population structure, was also used in assigning putative populations. The program takes allele frequencies into consideration and can be used to determine a likely number of populations existing within a group of samples and to assign individuals to these populations.

II.2.7.2 Intrapopulation Variation

II.2.7.2.1 Mitochondrial DNA

All analysis of mtDNA variation was based on the 171 bp consensus sequence obtained for all samples. Levels of intrapopulation variation were estimated as the number of haplotypes per population and as levels of nucleotide diversity (π). Nucleotide diversity is the probability that any two chosen homologous nucleotides within a sample are different (Nei 1987), and was calculated using ARLEQUIN 2.0 (Schneider et al. 2000).

II.2.7.2.2 Microsatellites

Measures of intrapopulation variation based on the five microsatellite loci used in this study are reported for each population by locus as the number of alleles, allelic richness, and levels of observed and expected heterozygosity. Allelic richness is a measure of the number of alleles corrected for sampling intensity using a rarefaction method designed by Hurlbert (1971) for measures of species diversity and applied to population genetics by (El Masoudik and Petit 1996; Petit et al. 1998). Allelic richness was determined by FSTAT 2.9.3.2 (Goudet 2001) with an adjusted sample size of $n = 19$. Levels of heterozygosity, as well as analysis of heterozygote deficiency and excess, were completed in ARLEQUIN 2.0 (Schneider et al. 2000) using Fisher's exact test and the Markov-chain method (Guo and Thompson 1992) (100,000 iterations, 5,000 dememorization steps, sequential Bonferroni correction applied).

II.2.7.3Interpopulation Comparisons

II.2.7.3.1 Mitochondrial DNA

Reported measures of population differentiation based on the mtDNA data include discussion of private and shared haplotypes between populations, as well as pairwise population ΦS_T and D_A values. D_A is a useful measure of genetic distance because it reports average numbers of pairwise differences between populations corrected for pairwise differences within populations ($D_A = D_{XY} - \left(\frac{D_X + D_Y}{2}\right)$) where D_{XY} is the average number of pairwise differences between populations, and D_X and D_Y are the average number of pairwise differences within the populations being compared (Nei 1987; Nei and Li 1979). Both ΦS_T and D_A were figured using methods implemented in ARLEQUIN 2.0 (Schneider et al. 2000) and significance was tested using 1,000 non-parametric permutations. Both measures were estimated using the Kimura 2-parameter genetic distance model (Kimura 1980). Kimura's model takes into consideration the possibility of multiple substitutions occurring at one site, as well differences in the frequencies of transitions and transversions, but does not account for variation in mutation rate along the length of the sequence as Tamura and Nei's (1993) model does. The short sequence fragment used in this study does not show a great amount of variation in number of polymorphic sites along the length of the sequence so it was determined that Kimura's model was most appropriate.

II.2.7.3.2 Microsatellites

Relationships between populations as shown through analysis of the microsatellite markers are presented as pairwise F_{ST}, Rho_{ST}, and $(\delta\mu)^2$ values. The measure of F_{ST} is

based on simple allele frequencies and was calculated using FSTAT 2.9.3.2 (Goudet 2001). The methods of Weir and Cockerham (1984) were used by this program and significance was tested by permutation procedures. F_{ST} is based on the Infinite Alleles Model (IAM) in which all new mutations are assumed to yield a new allele. However, the accepted mode of mutation for microsatellites, where strand slippage during replication leads to the addition or subtraction of one or more repeat units (Schlotterer and Tautz 1992), suggests that high levels of homoplasy exist in these alleles. Therefore, genetic distance measures based on the IAM model have been hypothesized to underestimate levels of genetic distance between populations (Goldstein et al. 1995a; Rousset 1996; Slatkin 1995). The Stepwise Mutation Model (SMM) takes into account the mode of mutation of microsatellites and genetic distance measures based on this model, such as Rho_{ST} and $(\delta\mu)^2$, have been developed. Rho_{ST} is a modification of Slatkin's (1995) R_{ST} that uses a weighting scheme based on changes of allele size proposed by Goodman (1997) and was calculated using R_{ST} Calc 2.2 (Goodman 1997). Goldstein et al. (1995b) developed $(\delta\mu)^2$, which is a measure that is independent of sample size, and this measure was also calculated for this study by R_{ST} Calc 2.2 (Goodman 1997). Significance levels for both Rho_{ST} and $(\delta\mu)^2$ were based on 1,000 permutations. Genetic distance measures based on both the IAM and SMM models were calculated here because although the SMM is based on the proposed mode of mutation for microsatellites and has therefore been claimed to be a superior measure (Rousset 1996), Goldstein et al. (1995b) suggested that IAM based measures may be better suited for defining relationships among closely related populations.

An assignment test, as performed in GeneClass2 (Piry et al. 2000), was completed to assess the uniqueness of the Flixborough samples as compared to modern animals.

Assignment was done using a likelihood-based Bayesian method (Rannala and Mountain 1997), and Monte-Carlo resampling was used to calculate probabilities of assignment to each population as described in Paetkau et al. (2003) (1,000 simulated individuals, α = 0.01).

II.2.7.3.3 Correlation of Genetic and Geographic Distances

To determine whether geographic distances between populations were able to explain the pattern of genetic differentiation seen by measures of F_{ST}, RhoST and $(\delta\mu)^2$, these distance measures were plotted against geographic distances measured as the shortest route by water between each population pair. The residuals of this linear regression were then correlated with the geographic distance measures and significance was tested using a Mantel test (Mantel 1967). The proportion of variation in genetic distances that was explained by geographic distance was reported as r^2 and significance was evaluated by 1,000 permuations as implemented in ARLEQUIN 2.0 (Schneider et al. 2000). Plots of geographic versus genetic distances and of geographic distance versus residuals were visualized using FSTAT 2.9.3.2 (Goudet 2001).

II.2.7.4 Phylogenetic Analysis

Evolutionary relationships between populations were investigated by phylogenetically comparing individual mtDNA haplotypes using both the neighbor-joining (Saitou and Nei 1987) and maximum parsimony methods as implemented in PAUP 4.0b10 (Swofford 1997). For both methods, majority-rule consensus trees rooted with a homologous killer whale (*Orcinus orca*) sequence (from Natoli et al. 2004) were generated using 1,000 bootstrap replications (Felsenstein 1985) with retention of branches supported

at a level of 50% or greater. Transition-transversion ratios based on observed levels of 6:1 were set, and the distances for the neighbor-joining tree were based on the Kimura 2-parameter distance model (Kimura 1980).

Chapter III – Results

III.1 Ancient DNA

III.1.1 Protocol Optimization

III.1.1.1 Sampling, Digestion, and DNA Extraction

Several protocols for aDNA extraction have been published that are designed to isolate as much DNA as possible while leaving behind the large amounts of PCR inhibitors often found in these samples. Two procedures were initially used in this study. Both protocols used a high EDTA/proteinase K digestion buffer to decalcify materials. From here, one protocol (from Ana Topf pers. comm.) used standard phenol-chloroform extraction methods (Sambrook et al. 1989) coupled with binding of DNA molecules to silica beads, while the second protocol (from Yang et al. 1998) used filterization of the digest through silica-based Qiagen QIAquick PCR Purification Kit™ columns.

Initial extractions using both protocols were performed on bones of recently deceased bottlenose dolphins obtained from strandings over the last 10 years. The success of the extraction procedures was assessed by their ability to yield extracts from which a 240 bp mtDNA product could be amplified. This assessment by PCR was necessary because ancient samples do not yield enough DNA to be visualized by standard ethidium bromide stained agarose gel electrophoresis. Both extraction protocols performed well with modern materials, yielding successful amplification of mtDNA in all samples attempted.

An extraction using both protocols on the same digest of three archaeological samples from Flixborough was then performed. One positive mtDNA amplification was achieved using the extracts obtained by the QIAquick™ method, while the phenol-chloroform based method yielded no successful products. Due to these results, the

QIAquick™ method was chosen for use throughout the rest of the study. Additionally, this method was preferred because the QIAquick™ columns do not release any DNA molecules over 10 kb (kilobasepairs) during elution, and therefore would trap large contaminating modern DNA molecules.

Once the DNA extraction method was chosen, the digestion of samples was further optimized to make the isolation more successful. In the first 28 extractions performed, volumes ranging from 3 to 5 mL of digestion buffer were added to the sample powder, dependent on the amount of bone powder (see Materials and Methods for details), and the buffer contained 0.133 mg/mL proteinase K (per Matisoo-Smith et al. 1997; Yang et al. 1998). Only 28.6% of samples (8 of 28) yielded mtDNA amplification products under these digestion conditions. Digest volume was then increased to range up to 8 mL because a significant amount of undigested solids still remained in some samples, and the proteinase K concentration was increased to 0.333 mg/mL. Following these changes, 78.3% of samples extracted (36 of 46) successfully gave mtDNA amplifications, some of which had failed to yield products in extractions done before the digest volume and proteinase K concentration had been altered. The success of DNA extraction did not vary significantly ($\chi^2 = 5.68$, df = 5, $p < 0.900$) with the approximate amount of bone powder available for extraction. This was investigated by the amount of digest buffer added to the sample. The details of digest volumes and extraction success for each sample are given in Table 1 of Appendix B.

III.1.1.2 PCR Amplification

Very little optimization of the PCR protocol for most loci occurred once data collection had begun in this study. Standard recommendations for PCR amplification of

degraded samples, including the use of a hot start polymerase, bovine serum albumin (BSA), a larger concentration of each primer, and increased cycle numbers, as well as selection of primers that yield a small product size, were taken into consideration before work commenced. The optimal $MgCl_2$ concentration and annealing temperature was determined for each set of primers by titrations performed on modern DNA samples, using aDNA conditions, before trial on the Flixborough samples (see Table 2.2 for conditions used with each primer). Still, some titration on aDNA samples was required for microsatellite locus D18. The initial conditions chosen for aDNA, 1.3 mM $MgCl_2$ and a 62°C annealing temperature, did not amplify the locus in the Flixborough samples, as only 1 of 19 in the first group attempted yielded a PCR product. However, further titrations using modern samples amplified under conditions necessary for aDNA work showed that successful reactions were also possible using 1.8 mM $MgCl_2$ and a 62°C annealing temperature. Amplification of the Flixborough samples under these conditions yielded 21 positives for 28 reactions in the first group tried. These conditions were then used for the rest of the study.

The amount of DNA extract used per reaction was the only variable in PCR reactions that was changed over all microsatellite loci during this study. Due to concern about the amounts of extracts remaining near the end of the laboratory work, the volume of extract used per 25 µL reaction was decreased from 5 µL to 3 µL. This did not affect the amplification success rates; however, concern was raised that many homozygote genotypes were being obtained. It was therefore deduced that the decrease in DNA concentration may have been contributing to allelic dropout rates, and the DNA amount was returned to 5 µL after trial in just 95 amplifications.

III.1.2 Mitochondrial DNA Sequencing

Extraction and amplification of mtDNA was attempted in 68 specimens from the Flixborough collection. Although some required a second attempt at extraction before a product was achieved, mtDNA was amplified in 47, giving a 69.1% success rate for DNA extraction/mtDNA amplification.

However, readable sequences were not always obtained from direct sequencing of the PCR products. Figure 3.1 shows two chromatograms produced by sequencing of the Flixborough products that demonstrate common problems seen when directly sequencing aDNA PCR products. The precise overlapping of peaks for thymine and cytosine, seen in position 136 of chromatogram A in Figure 3.1, is a feature of aDNA direct sequences and results from hydrolytic deamination of cytosine, which yields a thymine residue (Lindahl 1993). Other unclear base calls, also evident in chromatogram A (positions 130, 133 and 137), may have arisen due to miscoding lesions on some molecules (Hansen et al. 2001) or contamination within the extract that yielded two overlapping sequences differing at polymorphic sites. Some difficult base calls were also likely due to secondary structures within the DNA molecule. In some cases, adjustments of chromatogram peak heights in Sequencher 4.0 (Gene Codes Coporation) and comparisons with other ancient and modern sequences, or sequencing with the opposite primer were able to clarify ambiguous bases. Most often though, another amplification or sequencing reaction was needed to clear up the position. Unreadable sequences where many bases could not be determined with certainty, such as that seen in chromatogram B of Figure 3.1, were obtained occasionally. These required a new PCR reaction and sequencing.

New amplifications from an extract often allowed clearer interpretation of sequences that showed difficulties. Occasionally though, only a new extract would yield an acceptable sequence. Many specimens required multiple extractions and/or PCR

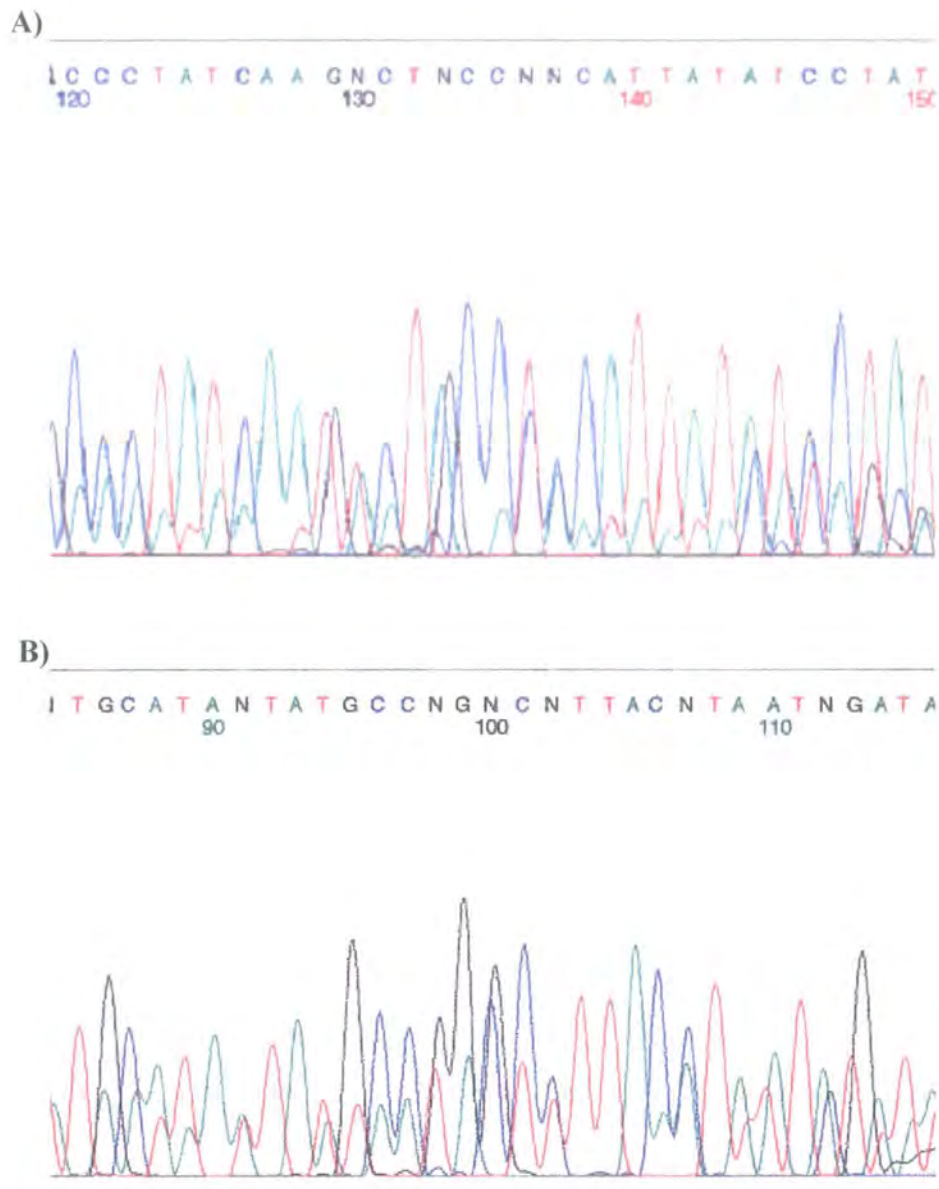

Figure 3.1. Chromatograms of mtDNA sequences obtained from Flixborough specimens exhibiting common difficulties observed with aDNA direct sequencing. Features of the chromatogram are as described in Figure 2.4. A) Overlapping cytosine and thymine peaks can be seen at position 136, likely resulting from hydrolytic deamination of cytosine to thymine in some molecules. Other unclear peaks are present at positions 129,130, 133, and 137. B) Exhibits an aDNA sequence with areas of indeterminate sequence from positions 98-102 and 111-115 that required a repeat of PCR amplification and sequencing to clarify these regions.

amplifications before an acceptable sequence could be obtained, but eventually 40 samples yielded readable mtDNA sequence results. However, the authenticity of all aDNA results must be proved by duplication and one sample could not yield a sequence from repeated extractions, and was therefore not used in the analysis. All 39 samples that were included in the results produced identical sequences from two independent extractions. Overall success rates for mtDNA sequencing in the Flixborough samples can be determined in that 83.0% of samples that yielded mtDNA PCR products gave usable, duplicated sequences. However, only 57.3% of all samples from which DNA extraction was attempted gave repeatable, reliable sequences that could be used in analysis. The details of the numbers and results of extractions attempted on each specimen as well as information about sequences acquired using both mtDNA primers can be found in Table 1 of Appendix B.

Variation in the ability to recover replicated sequences among differing bone fragment types was investigated, but no significant relationship was found ($\chi^2 = 6.44$, df = 7, $p < 0.9$). The success of DNA recovery from teeth compared to all bone samples was also studied and teeth performed significantly worse than bone samples ($\chi^2 = 5.99$, df = 1, $p < 0.025$). Table 3.1 lists the proportion of each sample type that yielded duplicate sequences from independent extractions. All sequences verified the morphological identification of the samples as *Tursiops truncatus*.

III.1.3 Microsatellite DNA

Amplification of microsatellites was only attempted in Flixborough samples that had yielded repeatable mtDNA sequences. Still, amplification of these markers proved much more difficult. Amplification efficiencies varied significantly by locus ($\chi^2 = 34.34$, df = 4, $p < 0.001$), ranging from 55.0% to 77.8%. Table 3.2 gives average PCR product

Table 3.1. Success rates by type of sample for extraction/mtDNA sequencing and amplification of microsatellite loci in ancient specimens. Amplification of microsatellite loci was only attempted in samples that yielded mtDNA sequences that had been duplicated in independent extractions.

Sample Type	Number of Samples Extracted	Samples Yielding Duplicated mtDNA Seq.	Successful Proportion for mtDNA Sequencing	Successful Proportion of Microsatellite Amplifications
Caudal Vertebra	8	5	0.625	0.773
Lumbar Vertebra	13	7	0.538	0.626
Thoracic Vertebra	10	8	0.800	0.703
Rib	14	10	0.714	0.643
Cranial Fragment	6	5	0.833	0.421
Scapula	2	1	0.500	0.440
Rostrum	4	1	0.250	0.733
Sternum	1	1	1.000	0.231
Tooth	7	1	0.143	0.333

Table 3.2. PCR product size range and amplification efficiencies within the Flixborough samples for all microsatellite loci.

Locus	PCR Product Size Range	Number of Attempted Amplifications	Proportion of Successful Amplifications
$TtruAAT_{44}$	82 – 106	124	0.726
D22	111 – 135	218	0.550
D08	93 – 123	225	0.551
MK8	95 – 115	210	0.576
D18	72 - 106	167	0.778

size and amplification efficiencies for each locus. The two loci showing the highest amplification efficiencies produced the shortest average PCR products, but the proportion of successful amplifications did not strictly correlate with PCR product size for the remaining loci.

Variation was also great between samples. Microsatellite amplification efficiencies for all Flixborough samples can be seen in Table 3.3. On average, 63.1% of attempted microsatellite amplifications yielded a visible product on an agarose gel, but efficiencies ranged from as low as 15.8% to 100% in different bone fragments. Efficiencies also varied significantly by bone type ($\chi^2 = 58.5$, df = 7, $p < 0.001$), as all types of vertebrae, ribs and the rostrum fragment gave superior results. Bone samples also performed significantly better in microsatellite amplifications than did the one tooth sample ($\chi^2 = 9.85$, df = 1, $p < 0.005$). Additionally, although the tooth specimen did yield some positive microsatellite amplifications, not a single locus gave a duplicated genotype that could be used for data analysis. The very small sample size of one for the specimen types of rostrum, scapula, sternum and tooth must be taken into consideration in interpretation of these results. Details of microsatellite amplification efficiencies by specimen type can be seen in Table 3.1.

The acceptance of an observed peak on the chromatograms produced by ABI Genotyper™ as a possible allele was practiced as described in the Materials and Methods. Microsatellites from the Flixborough samples more often showed troublesome peaks than amplifications from modern samples. Common problems included low peak amplitude, as peaks lower than 50 units were not acceptable, and poor structure, such as the lack of the signature stutter peaks for the locus or lumpy, poorly shaped peaks. Examples of poorly

Table 3.3. Amplification efficiencies over all microsatellite loci for each Flixborough sample.

Sample	**Number of Attempted Amplifications**	**Proportion of Successful Amplifications**
1e	25	0.560
1h	27	0.778
1j	22	0.364
2a	26	0.731
2b	28	0.714
4	29	0.690
5a	26	0.808
5b	20	1.000
6a	30	0.733
6d	24	0.542
6e	21	0.952
7	25	0.640
8c	22	0.955
9b	29	0.724
11c	28	0.643
11d	23	0.304
16	27	0.333
19b	19	0.158
20b	19	0.474
21b	16	1.000
23a	24	0.667
26	24	0.250
30	25	0.440
32	25	0.520
37	21	0.952
38	28	0.714
39	21	0.810
40b	26	0.231
41	24	0.833
42	22	0.727
44l	27	0.630
45c	25	0.520
45d	23	1.000
46	22	0.864
47	30	0.167
50e	23	0.261
51	25	0.480
52b	16	1.000
58	25	0.440

shaped peaks originating from amplifications in ancient samples can be seen in Figure 3.2 alongside acceptable, well-formed peaks.

Once an allele (or peak) observed for an aDNA specimen was deemed reliable, the acceptance of the genotype for use in analysis was still dependent upon additional criteria for replications based on the multiple-tubes approach (Navidi et al. 1992). The occurrence of false alleles, which was obvious when three or more alleles were recorded over replicates for a single sample, was noted 26 times. Twelve of these occurrences could be explained by Taberlet et al.'s (1996) hypothesis of slippage during early steps of the PCR reaction leading to peaks one repeat smaller than the true allele showing up at nearly equal amplitudes to the true allele. With the conditions for acceptance set out by Taberlet et al. (1996) and multiple replicates, all but one of these genotypes was accepted. However, the remaining 14 may have been due to sporadic contamination and in only one of these cases was sufficient replicates (12) available to verify the true genotype. In all other cases the genotypes were not used in the analysis. All false alleles noted in this study occurred in loci D08, D18, D22 and MK8, all of which contain dinucleotide repeat sequences. In locus $TtruAAT_{44}$, a trinucleotide repeat marker, no false alleles were observed.

Criteria based on the multiple tubes approach (Navidi et al. 1992) were used to account for problems of allelic dropout in diploid nuclear markers of aDNA. The three data sets that were compiled based upon stringency for the acceptance of homozygote genotypes varied greatly in the number of genotypes that were included. The least stringent criteria of accepting all homozygote genotypes that had been obtained with a minimum of two duplications showing no other alleles contained a total of 59 homozygotes, that which required levels of reliability suggested by Gagneux et al. (1997) left 43 homozygotes in the analysis, but the most stringent criteria used, Taberlet et al.'s (1996) reasoning, allowed only 11 homozygotes to be accepted. Levels of observed

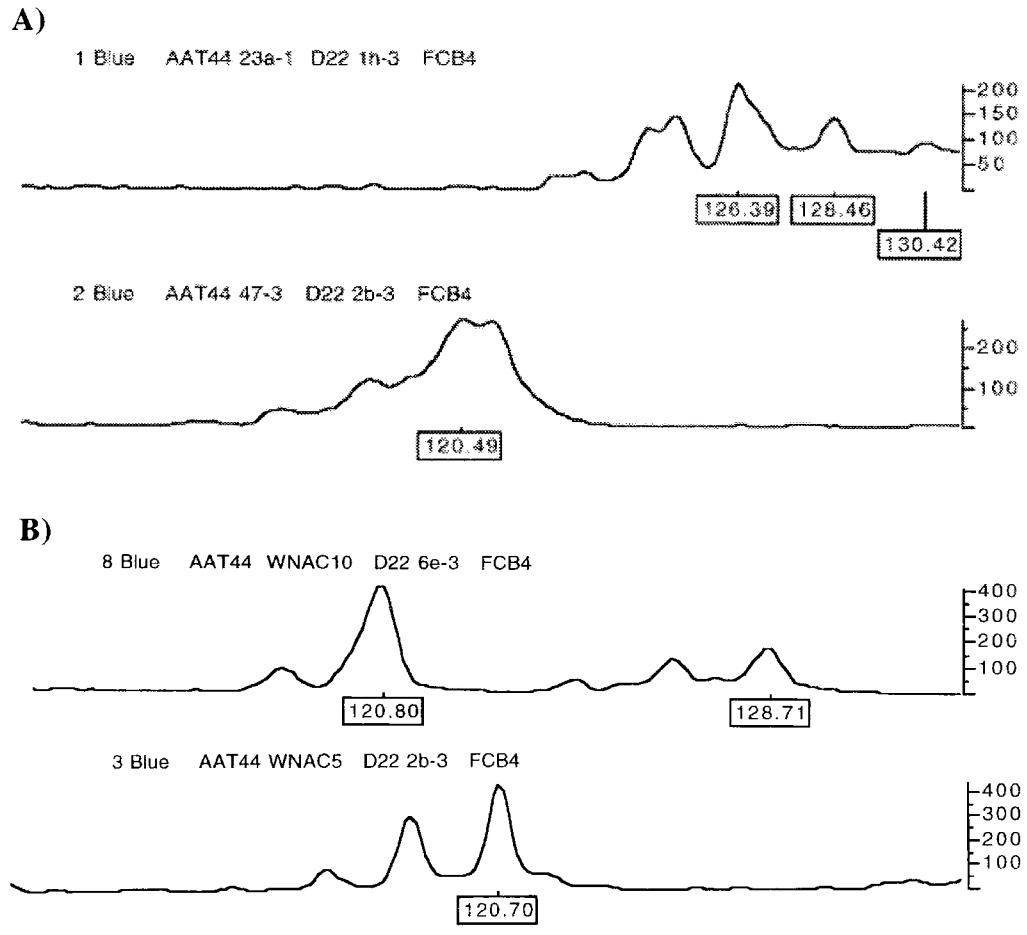

Figure 3.2. Microsatellite Genotyper™ chromatograms illustrating poorly shaped and acceptable peaks encountered for one locus, D22, amplified in aDNA. Peak amplitudes can be judged from the scales present to the right of each lane and PCR product sizes are indicated in the boxes under peaks. A) Both show examples of poor peak structure, as irregularly shaped (lane 1) and lumpy (lane 2) peaks were treated with caution. B) Both show clearly defined peaks that exhibit the signature structure of this dinucleotide repeat locus.

heterozygosity obviously differed between these data sets and this can be seen in Table 3.4. The data set based on the least stringent criteria showed a heterozygote deficit in all loci (statistically significant in two), while that which followed the most stringent criteria actually showed a heterozygote excess at all loci (also significant in two). Surprisingly though, initial analyses run with each data set showed no meaningful differences in measures of population differentiation. Relationahip trends to other populations, as defined by F_{ST} values between Flixborough and other populations, remained the same with all three data sets, and judgments of significant differentiation also did not change. Furthermore, measures of intrapopulation variation for Flixborough varied little between data sets. Allele number was the same in all data sets and allelic richness deviated only slightly as shown in Table 3.5. Therefore, all further analysis reported in this thesis was done with the most inclusive data set.

III.1.4 Authenticity of Ancient DNA Results

Confidence in the aDNA results reported in this thesis was gained by adherence to several of the verification criteria for aDNA reviewed in Cooper and Poinar (2000). First, all results were replicated in independent extractions. Further details for their acceptance were discussed in the preceding sections. Results were not confirmed by duplication in an independent laboratory, but this is only deemed critical in studies of humans or when novel results are obtained (Cooper and Poinar 2000)

Several features of the molecular behavior of the Flixborough extracts lend credibility to the results, as they are the expected behavior of aDNA molecules. The low intensity of PCR products obtained from the Flixborough samples in comparison with positive controls run in parallel for both mtDNA and microsatellite amplifications are shown in Figure 3.3. This points to a decreased number of molecules at initiation of the

Table 3.4. Tables showing observed and expected heterozygosities and assessing deviation from Hardy-Weinberg equilibrium for each of the three data sets formed from microsatellites typed in the Flixborough population. A) Includes all genotypes duplicated a minimum of two times. B) Incorporates all genotypes accepted by statistical criteria suggested in Gagneux et al. (1997). C) Consists of genotypes deemed acceptable by criteria based on Taberlet et al. (1996). *P*-values that are statistically different from zero after sequential Bonferroni correction (α=0.05) are marked with an asterisk (*). H_o = observed heterozygosity, H_e = expected heterozygosity.

A)

Locus	Number of Genotypes	H_o	H_e	*p*-value
TtruAAT$_{44}$	33	0.485	0.560	0.050
D22	22	0.454	0.511	0.172
D08	20	0.650	0.671	0.007*
MK8	23	0.609	0.706	0.008*
D18	29	0.517	0.564	0.052

B)

Locus	Number of Genotypes	H_o	H_e	*p*-value
TtruAAT$_{44}$	33	0.485	0.560	0.053
D22	22	0.454	0.511	0.175
D08	16	0.813	0.708	0.006*
MK8	18	0.778	0.713	0.001*
D18	22	0.682	0.641	0.058

C)

Locus	Number of Genotypes	H_o	H_e	*p*-value
TtruAAT$_{44}$	17	0.941	0.749	0.050
D22	12	0.833	0.620	0.472
D08	13	1.000	0.735	0.000*
MK8	17	0.824	0.718	0.003*
D18	20	0.750	0.686	0.047

Table 3.5. Allele number and allelic richness values for the three Flxborough data sets based on varying criteria for acceptance of homozygote genotypes. Number of genotypes is the number of samples for which a reliable genotype was obtained.

Locus	All Duplicated Homozygotes	Data Set Based on Gagneux et al. (1997)	Data Set Based on Taberlet et al. (1996)
TtruAAT$_{44}$			
No. of genotypes	33	33	17
No. of alleles	5	5	5
Allelic richness	4.500	4.339	4.681
D22			
No. of genotypes	22	22	12
No. of alleles	4	4	4
Allelic richness	3.998	3.983	4.000
D08			
No. of genotypes	20	16	13
No. of alleles	4	4	4
Allelic richness	4.000	4.000	4.000
MK8			
No. of genotypes	23	18	17
No. of alleles	4	4	4
Allelic richness	3.973	3.990	3.920
D18			
No. of genotypes	29	22	20
No. of alleles	5	5	5
Allelic richness	4.847	4.913	4.787

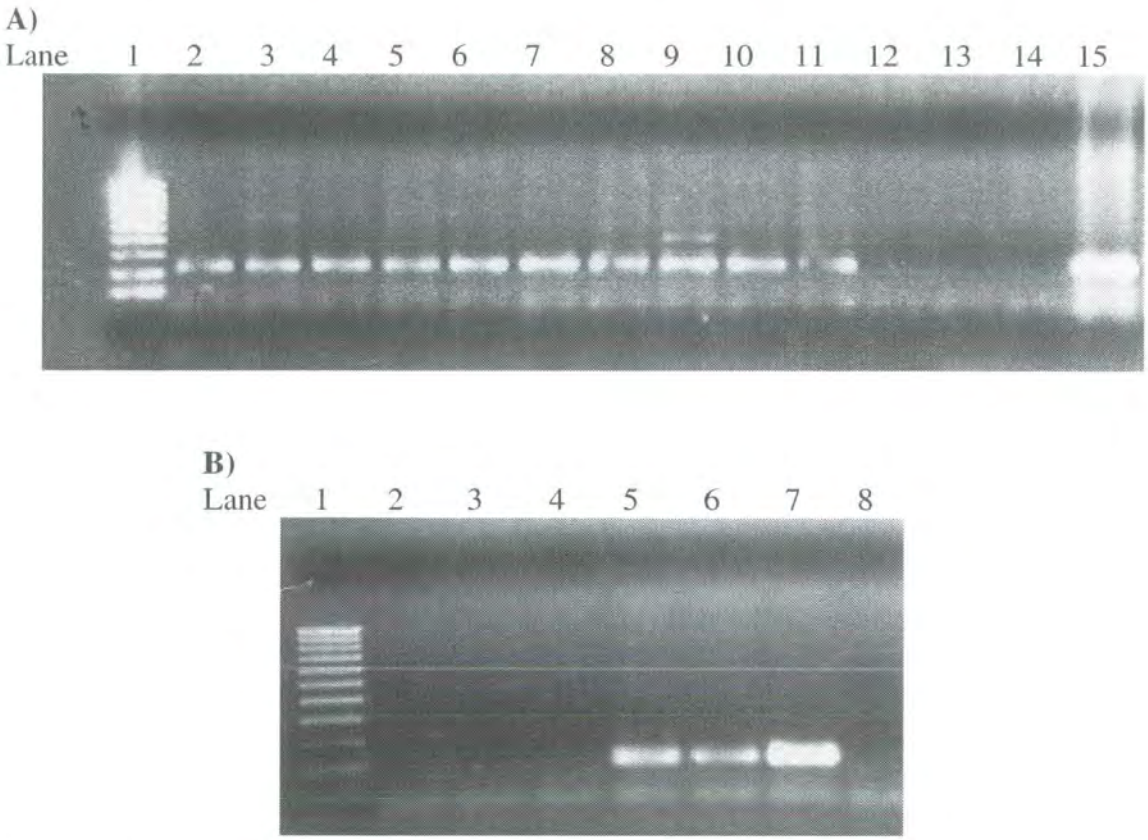

Figure 3.3. Agarose gel electrophoresis of A) mtDNA and B) microsatellite PCR products displaying the increased intensity of positive PCR controls using modern DNA that were amplified and ran on agarose gels in parallel with aDNA reactions. Lane assignments: A) 1. Sigma 100bp low DNA size marker, 2-12. ancient mtDNA amplifications, 13. Extraction control, 14. PCR negative control, 15. PCR positive control. B) 1. Sigma 100bp low DNA size marker, 2-6. aDNA microsatellite amplifications, 7. Positive PCR control, 8. Negative PCR control.

reaction. The common presence of many difficult base calls, such as occurrences of overlapping cytosine and thymine peaks, and the occasional return of unreadable sequences discussed above and demonstrated in Figure 3.1, are also indicative of sequences from ancient, degraded templates. The molecular behavior during work with the microsatellite loci also gives evidence of the ancient nature of the extracts, simply by the difficulty in amplification of these markers. Furthermore, amplification of EV37 (Valsecchi and Amos 1996), a microsatellite locus that gives products of greater than 200 bp in length in bottlenose dolphins, was attempted in 10 samples and not a single reaction yielded a product. If longer, contaminating modern DNA molecules were present in the extracts, products would have been obtainable with this locus. Indicative of allelic dropout, levels of heterozygosity are lower than expected for all microsatellite loci in the Flixborough population (significantly so for two loci) without strict criteria for acceptance of homozygotes being applied (see Table 3.4). Allelic dropout was also commonly noted by initial genotypes of samples showing only one allele, that upon duplication yielded heterozygote genotypes.

The results obtained for the Flixborough specimens also make sense in the context of this study and the phylogeny of bottlenose dolphins. Both the mtDNA and microsatellites show that the Flixborough animals are most closely related to populations around the UK and the eastern North Atlantic (see following results), which is a reasonable result. The lack of variation in the mtDNA sequences from Flixborough (see following results) was initially a concern, as it was feared that wholesale contamination might have led to the overwhelming occurrence of one haplotype. However, the variation in microsatellite loci alleviated this fear and clarified that distinct animals were being studied. In fact, microsatellite data showed that three Flixborough samples (6a, 6d and 6e), initially

hypothesized to be from one animal, had distinct genotypes over the five loci and were therefore from different animals.

The published retrieval of DNA from goose bone fragments excavated at Flixborough (Barnes et al. 2000) also gives confidence in the authenticity of the results reported here because it suggests that conditions at the site were conducive to DNA survival. However, no quantification of levels of DNA, by-products of DNA degradation, or other biomolecules present in the Flixborough bottlenose dolphin samples was completed for this study.

III.2 Determination of Putative Populations

Separation by large geographic distance made division of some samples for analysis straightforward, while previously published accounts of differentiation were considered in separation of some less likely distinct or even parapatric groups (Hoelzel et al. 1998, West North Atlantic pelagic and coastal; Natoli et al. 2004, Mediterranean Sea, Black Sea, African *truncatus* and *aduncus*; Wang et al. 1999, Chinese *truncatus* and *aduncus*). However, some population divisions were not so easily defined. For eastern North Atlantic samples from waters around Portugal, Galicia, Ireland, and the United Kingdom, the study of Hardy-Weinberg equilibrium within population groupings and pairwise population F_{ST}'s between populations using microsatellite data, as well as the use of *Structure* 2.0 (Falush et al. 2003; Pritchard et al. 2000), led to the putative population groupings used.

Samples from Portugal were only used in the microsatellite analyses, but they were grouped with the Galicia samples into a single population because a pairwise F_{ST} did not show significant differentiation ($F_{ST} = 0.005$, $p = 0.615$), and there was no significant

departure from Hardy-Weinberg equilibrium at any locus when the two sample groups were combined (see Table 3.8). *Structure* also gave no indication of population separation as α varied wildly from −1.5 to 1.0 throughout multiple runs, and all individuals were shown to be admixed in nearly equal proportions. These results are both signals that no real differentiation exists in a group according the manual for *Structure*.

Population groupings within the United Kingdom and Ireland samples were more difficult to define. Initial analyses used groupings suggested by reports of resident populations in the Shannon Estuary (Berrow et al. 1996), Moray Firth (Hammond and Thompson 1991; Wilson et al. 1997), Outer Hebrides (Grellier and Wilson 2000) and Cardigan Bay (Grellier et al. 1995). However, pairwise F_{ST} values, presented in Table 3.6, showed that not all of these groups were significantly differentiated. Yet, samples from the northeast of Scotland and Ireland were strongly differentiated from most groups, as were the Flxiborough samples. The southeast England samples were not differentiated from any group, but the small sample size of three must be considered in interpretation of these results. Various combinations of sample groupings based on geographical proximity were then tested by analysis of F_{ST} values and deviation from Hardy-Weinberg equilibrium. It was found that the sample group from around the Moray Firth in the northeast of Scotland was significantly differentiated from all other animals in waters around the United Kingdom and Ireland as a group (see Table 3.10). Studies of Hardy-Weinberg equilibrium supported this division as the putative population created by grouping all samples outside of the Moray Firth showed a statistically significant heterozygote deficiency at only one locus (D18, $p = 0.000$) (see Table 3.8). It is not thought that this deficiency is a result of Wahlund's effect, but is perhaps more likely due to inbreeding as there is very little variation for D18 within this group, and observed

Table 3.6. Pairwise F_{ST} values for microsatellite data for putative population groups around the United Kingdom and Ireland based on published accounts of resident populations. Estimates showing p-values significantly different from zero at the $p < 0.05$ level are marked with an asterisk (*). Those with p-values significant after Bonferroni correction ($p < 0.001$) are marked with a double asterisk (**). Sample abbreviations are FLIX=Flixborough, NES=Northeast Scotland, WS=West Scotland, IRE=Ireland, WAL=Wales and SEE=Southeast England. Sample size (n) is given under the sample abbreviations on the top row. Results for one locus of the five used, D08, in 27 modern animals are from Natoli et al. (2004).

	FLIX $n = 38$	**NES** $n = 27$	**WS** $n = 7$	**IRE** $n = 13$	**WAL** $n = 13$	**SEE** $n = 3$
FLIX						
NES	0.163**					
WS	0.071*	0.084**				
IRE	0.244**	0.110**	0.145**			
WAL	0.138*	0.057**	0.004	0.057*		
SEE	0.126	-0.016	-0.043	0.023	-0.081	

heterozygosity over the group for this locus would certainly be lowered by the samples from Ireland where all but 2 of the 13 samples were homozygous for the same allele. *Structure* gave a probability of 1.0 for a K of 2, but this was not trusted to be a reliable estimate of K because all values of ln $\Pr(X \mid K)$ were fairly distant from one another, meaning that the smallest ln $\Pr(X \mid K)$ would necessarily show a $\Pr(K)$ of 1.0 while all others were essentially zero. The plateau in ln $\Pr(X \mid K)$ values that is a suggested signal of arrival near a reasonable K value was not observed. The bar plot produced by *Structure* of individuals and estimates of their admixture using a K value of three is presented in Figure 3.4. Most samples from the northeast of Scotland appeared as a fairly well defined group, however the samples from the remaining regions did not show a clear separation into differentiated groups. All the above indicators led to the separation of samples from around the United Kingdom and Ireland into two putative populations, one of animals from the Moray Firth and nearby in the northeast of Scotland, and one containing all other animals from around the British Isles.

III.3 Intrapopulation Variation

III.3.1 Mitochondrial DNA

Sequences of the 171 bp consensus fragment of the mtDNA control region available for both the Flixborough specimens and samples from the populations shown in Figure 2.3 gave a total of 73 haplotypes distinguished by 40 polymorphic sites. Although there were two regions of slightly higher variation, polymorphic sites were found throughout the sequence.

Table 3.7 gives the number of sequences *(n)* obtained for each population and the number of haplotypes found in each population, as well as indicating the population

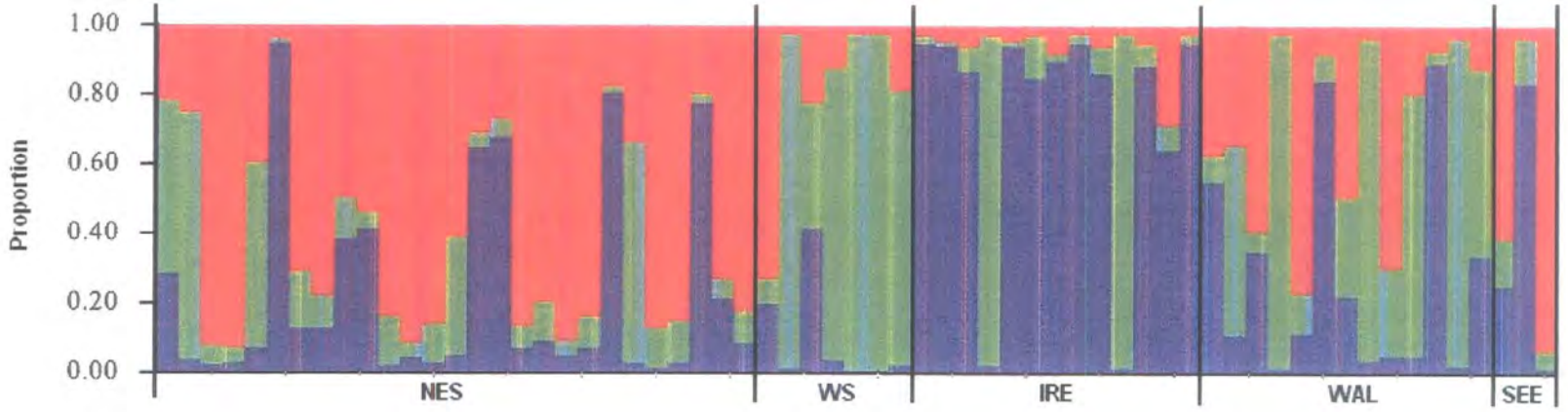

Figure 3.4. Bar plot produced by *Structure* (Falush et al. 2003; Pritchard et al. 2000) exhibiting assignments/admixtures of each modern sample from the UK and Ireland. Each bar represents an individual and each color a 'population.' The proportion of a bar shaded each color illustrates the proportion of each sample assigned to that 'population.' The *K* (population number) used here is three. The vertical black lines separate the sample groups from regions of the UK and Ireland. Abbreviations as are in Table 3.6.

Table 3.7. Number of mtDNA haplotypes and nucleotide diversity (π) with standard deviation for each population. Population abbreviations are FLIX=Flixborough, NES=Northeast Scotland, OUK=United Kingdom and Ireland (except northeast Scotland), GAL=Galicia, MS=Mediterranean Sea, BS=Black Sea, WNAP=Western North Atlantic Pelagic, WNAC=Western North Atlantic Coastal, GM=Gulf of Mexico, AT=African *truncatus*, SAA=South African *aduncus*, CHT=Chinese *truncatus*, CHA=Chinese *aduncus*. Sample size (*n*) for each population is given in parentheses after the abbreviation. The sources for all sequence data are reported in the final column.

Population	**Number of haplotypes**	**Nucleotide Diversity (π)**	**Source of Sequences**
FLIX (39)	2	0.002 +/- 0.002	*this study*
NES (28)	1	0.000 +/- 0.000	*7 Natoli et al. 2004;13 Parsons et al. 2002; 8 this study*
OUK (37)	11	0.021 +/- 0.012	*8 Natoli et al. 2004; 7 Parsons et al. 2002; 22 this study*
GAL (14)	9	0.024 +/- 0.014	*Natoli et al. 2004*
MS (34)	14	0.029 +/- 0.016	*Natoli et al. 2004*
BS (14)	5	0.030 +/- 0.018	*Natoli et al. 2004*
WNAP (25)	11	0.029 +/- 0.016	*Hoelzel et al. 1998*
WNAC (29)	6	0.010 +/- 0.006	*Hoelzel et al. 1998*
GM (14)	7	0.014 +/- 0.009	*Natoli et al. 2004*
AT (16)	5	0.020 +/- 0.012	*Natoli et al. 2004*
SAA (38)	5	0.004 +/- 0.003	*Natoli et al. 2004*
CHT (17)	9	0.022 +/- 0.013	*Wang et al. 1999*
CHA (19)	10	0.022 +/- 0.013	*Wang et al. 1999*

abbreviations used from here forward for discussion of mtDNA results and the source of all previously published sequences. Most notable from this data is the low number of haplotypes in the FLIX and NES samples. Despite having comparable, or in the case of FLIX a larger sample size (n = 39) than all other populations, FLIX and NES contain only two and one haplotypes, respectively, while all others include a minimum of five. Also notable for the Flixborough sample is that the two haplotypes found in FLIX differ by only two polymorphic sites and the population is dominated by one haplotype, as 36 of the 39 sequences were identical. Haplotype frequencies for mtDNA in FLIX and all other samples can be found in Table 2 of Appendix B. It can be seen from there that FLIX and NES are the only populations that do not contain private haplotypes.

The lack of variation in mtDNA in FLIX and NES is further indicated by the nucleotide diversity (π) values presented in Table 3.7. Nucleotide diversity values for FLIX and NES are the lowest reported for any populations and are an order of magnitude lower than all populations except SAA.

III.3.2 Microsatellites

Microsatellite loci, by their nature, are likely to show more variation within populations than mtDNA. Additionally, all microsatellites used in this study exhibited high levels of variation. Total numbers alleles of found over all populations were as follows: TtruAAT$_{44}$ = 9, D22 = 12, D08 = 14, MK8 = 10, D18 = 15.

Levels of diversity at Flixborough for the microsatellite loci used are lower than in all current day populations studied here. Table 3.8 shows several indicators of intrapopulation variation by locus for each sample, as well as the population abbreviations used in all discussion on microsatellite results, the sources for all data, and the number of

Table 3.8. Various indicators of intrapopulation variation based on microsatellite results presented for each population by locus. Sample size for each population (*n*) is given underneath the abbreviation. Number of genotypes is the number of samples from each population for which genotypes were successfully obtained at each locus. Number of private alleles is given in parentheses following the number of alleles. H_o and H_e stand for observed and expected heterozygosity, respectfully. An asterisk (*) marks all H_o values with *p*-values indicative of statistically significant heterozygote deficiency after application of a sequential Bonferroni correction (α=0.05). The source of data for each locus is given in the last column. Population abbreviations are FLIX=Flixborough, NES=North East Scotland, OUK=United Kingdom and Ireland (except northeast Scotland), MS=Mediterranean Sea, P+G=Portugal and Galicia, WNAP=West North Atlantic Pelagic, WNAC=West North Atlantic Coastal. (Table 3.8 continues on the following page.)

Locus	FLIX *n* = 38	NES *n* = 27	OUK *n* = 36	P+G *n* = 29	MS *n* = 30	WNAP *n* = 27	WNAC *n* = 27	Source of Data
TtruAAT$_{44}$								*this study*
No. of genotypes	33	27	31	28	29	27	27	
No. of alleles	5	6	7	8	8	7	4	
Allelic richness	4.500	5.111	5.837	7.471	7.295	6.826	3.407	
H_o	0.485	0.593	0.645	0.857	0.690	0.778	0.630	
H_e	0.560	0.606	0.720	0.795	0.816	0.781	0.535	
D22								*this study*
No. of genotypes	22	26	31	29	28	26	27	
No. of alleles	4	5	11 (1)	8	8	8	7 (1)	
Allelic richness	3.998	4.461	9.420	7.422	7.685	7.594	6.618	
H_o	0.455	0.500	0.742	0.759	0.571	0.808	0.852	
H_e	0.511	0.560	0.775	0.817	0.779	0.852	0.782	

Locus	FLIX $n = 38$	NES $n = 27$	OUK $n = 36$	P+G $n = 29$	MS $n = 30$	WNAP $n = 27$	WNAC $n = 27$	Source of Data
D08								*17 NES,10 OUK, and all P+G, MS, WNAP, WNAC, Natoli et al. 2004; remaining, this study*
No. of genotypes	20	27	29	29	29	26	27	
No. of alleles	4	3	8 (1)	8 (1)	9 (1)	11 (1)	7	
Allelic richness	4.000	2.994	6.620	7.388	8.157	9.786	5.813	
H_o	0.650*	0.407	0.379	0.724	0.793	0.808	0.667	
H_e	0.671	0.440	0.469	0.771	0.831	0.870	0.600	
MK8								*this study*
No. of genotypes	23	20	29	24	28	22	19	
No. of alleles	4	5	7	8	8	8	6 (1)	
Allelic richness	3.973	4.950	6.299	7.576	7.479	7.847	6.000	
H_o	0.609*	0.800	0.552	0.917	0.786	0.773	0.789	
H_e	0.706	0.685	0.787	0.824	0.832	0.832	0.713	
D18								*this study*
No. of genotypes	29	27	35	29	30	26	27	
No. of alleles	5	4	12 (2)	10	9	12 (1)	4	
Allelic richness	4.847	3.997	9.424	9.557	8.438	11.302	3.704	
H_o	0.517	0.556	0.514*	0.862	0.767	0.846	0.630	
H_e	0.564	0.664	0.728	0.887	0.853	0.901	0.650	

genotypes successfully obtained for each locus in each population. FLIX contains fewer alleles than all other populations for most loci, with the exception of two loci in both NES and WNAC. This is true despite the nearly equivalent number of genotypes obtained for Flixborough and other populations. Lower variability for microsatellite loci in the Flixborough population is further evidenced by the values of allelic richness. The lowest values are again seen in FLIX for all loci, once more with the exception of two loci in both NES and WNAC. As was the case with the mtDNA sequences, all populations except FLIX and NES show a private allele in at least one locus. Tables of microsatellite allele frequencies for all populations and loci can be seen in Table 3 of Appendix B.

Values of observed and expected heterozygosity are also shown in Table 3.8. Overall, the lowest heterozygosities are seen in the Flixborough sample and statistically significant heterozygote deficiencies are present in two loci in FLIX. These deficiencies, however, could very likely be due to allelic dropout within this sample. A statistically significant heterozygote deficiency only occurs elsewhere for locus D18 in OUK. The likely causes of this were considered in the discussion of determining population subdivisions. However, heterozygote excess occurs in one locus in NES, two loci for P+G and in four loci for WNAC, though none show *p*-values significantly different from zero. P+G and WNAP show the overall highest levels of heterozygosity.

III.4 Interpopulation Comparisons

III.4.1 Mitochondrial DNA

Analyses of interpopulation differentiation based on the mtDNA data must be interpreted with caution because the 171 bp fragment used is a quite short segment for consideration. This is evidenced by the fact that a study by Parsons et al. (2002) including

many of the same samples used here, reported two haplotypes that differed by one site for a 549 bp sequence in 15 animals from the Moray Firth. However, this polymorphism was removed upon trimming of the sequences to 171 bp for this study. Levels of differentiation for both FLIX and NES also will likely be affected by the low levels of variation present in both of these samples.

Despite the limitations of this analysis, haplotype frequencies point to some trends of differentiation as all populations except FLIX and NES show private haplotypes in the region studied. Furthermore, WNAC, GM, SAA, CHA and CHT have completely fixed differences, as no haplotype is shared with another population (see haplotype frequencies reported in Table 2 of Appendix B). Simple haplotype frequencies also point to a closer association between FLIX and some geographically close populations as the dominant haplotype in FLIX (found in 36 samples) is the same haplotype found in NES. This haplotype is also present in overwhelming numbers in OUK (found in 23 samples), and constitutes nearly half of the sequences in GAL (found in 6 samples). This same haplotype is present at lower frequencies in MS and AT. The less common haplotype at FLIX is found elsewhere in one western Scottish sample from OUK, as well as once in GAL and twice in AT.

Studies of levels of population differentiation as indicated by pairwise Φ_{ST} analysis of mtDNA sequences based on the Kimura 2-parameter genetic distance model are presented in Table 3.9. Significant differentiation of FLIX from all other populations except NES is demonstrated. The sample of animals from elsewhere around the United Kingdom and Ireland is the next most closely related to Flixborough, showing differentiation only at the $p < 0.05$ level, while all others are significant at the $p < 0.001$ level. Most populations are significantly differentiated from all others at the $p < 0.001$

Table 3.9. Φ_{ST} and D_A for pairwise population comparisons using mtDNA sequence data. Φ_{ST} values based on the Kimura 2-parameter genetic distance model are reported below the diagonal. Values that are statistically significant from zero at the $p < 0.05$ level are marked with an asterisk (*), those at the $p < 0.001$ level with two asterisks (**). When a Bonferroni correction is applied (α=0.05), $p < 0.00076$ is significantly different from zero. All values marked as being significant at $p < 0.001$ are also significant at this corrected level. D_A values are also based on the Kimura 2-parameter model and are reported above the diagonal. All are statistically significant at the $p < 0.001$ level, as well as the Bonferroni corrected level of $p < 0$ .00076. Population abbreviations and sources for previously published sequences are as given in Table 3.7.

	FLIX	NES	OUK	GAL	MS	BS	WNAP	WNAC	GM	AT	SAA	CHT	CHA
FLIX		0.008	0.278	0.531	1.978	3.059	2.389	5.429	6.044	2.391	6.224	4.670	10.832
NES	0.037		0.359	0.634	2.178	3.251	2.614	5.410	6.022	2.660	6.203	4.876	10.980
OUK	0.130*	0.145**		0.039	0.863	1.593	1.267	4.410	5.422	1.064	5.524	3.486	9.423
GAL	0.332**	0.352**	0.013		0.233	0.865	0.538	4.011	4.761	0.649	5.680	2.238	8.746
MS	0.440**	0.435**	0.168**	0.042		0.220	0.295	4.524	5.606	0.136	6.526	1.583	8.562
BS	0.664**	0.654**	0.288**	0.155*	0.042		0.911	4.944	6.158	0.405	7.289	2.227	9.334
WNAP	0.549**	0.544**	0.242**	0.106*	0.056*	0.157*		4.335	5.151	0.638	6.367	1.251	8.591
WNAC	0.868**	0.872**	0.622**	0.635**	0.565**	0.643**	0.592**		4.105	5.620	3.543	5.710	9.047
GM	0.877**	0.882**	0.623**	0.594**	0.560**	0.608**	0.567**	0.690**		6.727	5.539	4.536	6.004
AT	0.674**	0.688**	0.232**	0.150*	0.023	0.087	0.129*	0.719**	0.692**		7.435	2.453	9.197
SAA	0.933**	0.946**	0.729**	0.794**	0.706**	0.799**	0.743**	0.777**	0.836**	0.840**		7.440	9.382
CHT	0.780**	0.776**	0.491**	0.365**	0.251**	0.330**	0.223**	0.707**	0.585**	0.403**	0.826**		8.818
CHA	0.882**	0.876**	0.721**	0.689**	0.647**	0.673**	0.664**	0.786**	0.645**	0.714**	0.849**	0.696**	

level, however, there is a cluster of more closely related populations including GAL, MS, BS and AT that do not show significant divergence in some pairwise comparisons. Additionally, OUK is not significantly differentiated from GAL.

Table 3.9 also contains pairwise D_A values for all populations using the Kimura 2-parameter genetic distance model. All values are statistically significant from zero, so all populations were found to be differentiated. However, FLIX is again shown to be closest to NES and then to OUK and GAL, respectively, as was the case in the Φ_{ST} analysis. The same patterns of a grouping among GAL, MS, BS and AT seen in the Φ_{ST} analysis are again present, as is the close relationship between OUK and GAL.

III.4.2 Microsatellites

Differentiation between populations using microsatellite loci is suggested by the presence of private alleles in all populations except FLIX and NES and by the various statistics of population divergence presented below. Levels of population differentiation using the Infinite Alleles Model (IAM) are presented as pairwise F_{ST} values in Table 3.10. All populations are shown to be significantly different from all others in this analysis. Still, the lowest level of divergence indicated by F_{ST} values is between NES and OUK, as 4.9% of microsatellite genetic variation among these populations is due to genetic differences between the populations. Interestingly, much more of the variation between FLIX and both NES and OUK is due to differences between the populations (16.3% FLIX to NES and 14.0% FLIX to OUK). F_{ST} values also show FLIX to be most closely related to animals from P+G, while WNAP is less different from FLIX than NES. In a similar pattern to that seen in the mtDNA, OUK is quite closely related to P+G and a cluster of populations from P+G, MS and WNAP is apparent.

Table 3.10. Pairwise population F_{ST} values using microsatellite data. All values are significant at the Bonferroni corrected level of ($\alpha = 0.05$) $p < 0.003$. Population abbreviations and sources of data are as indicated in Table 3.8.

	FLIX	**NES**	**OUK**	**P+G**	**MS**	**WNAP**	**WNAC**
FLIX		0.163	0.140	0.098	0.153	0.144	0.210
NES			0.049	0.127	0.196	0.163	0.222
OUK				0.057	0.130	0.093	0.224
P+G					0.027	0.014	0.142
MS						0.046	0.160
WNAP							0.138

Microsatellite population differentiation values based on the Stepwise Mutation Model (SMM) are presented in Table 3.11. For both $(\delta\mu)^2$ and Rho_{ST}, all populations are significantly differentiated from all others with the exception of pairwise comparisons of MS and WNAP. Interestingly, $(\delta\mu)^2$ values indicate less distance between FLIX and both P+G and WNAP than either NES or OUK, although the FLIX to OUK relationship is significant at a less stringent level. Similarly, Rho_{ST} shows comparatively low levels for differentiation of FLIX from P+G and WNAP. As with F_{ST} values, it is notable that for both $(\delta\mu)^2$ and Rho_{ST}, OUK and NES are closer to each other than either population is to FLIX. Again as in F_{ST}, Rho_{ST} shows low levels of differentiation between OUK and P+G and both $(\delta\mu)^2$ and Rho_{ST} indicate a clustering of P+G, MS and WNAP.

Although the three genetic distances for microsatellites reported above are based on two different models of microsatellite evolution, common threads are apparent among the results reported by all of them. It is most notable that according to multiple microsatellite-based genetic distance values, the two modern-day putative populations from around the British Isles, NES and OUK, are genetically closer to each other than either is to FLIX, despite the close geographical proximity of FLIX to NES. Additionally, the close relationship of FLIX to both P+G and WNAP, which is on the other side of the Atlantic, as well as the clustering of P+G and MS with the geographically distant WNAP is indicated by all three microsatellite-based genetic distance measures.

An assignment test showed a strong identity for the Flixborough population. All but six individuals from Flixborough were assigned with the highest probability to the defined Flixborough population. Samples assigned to FLIX also showed comparatively low probabilities of belonging to any other group. Of those not assigned to FLIX, five individuals showed the highest probability of belonging to WNAP and one to OUK.

Table 3.11. Pairwise population $(\delta\mu)^2$ and Rho_{ST} values based on microsatellite data. $(\delta\mu)^2$ values are reported below the diagonal, Rho_{ST} above. Values significantly different from zero at the $p < 0.05$ level are marked with an asterisk (*). Values still significant after application of a Bonferroni correction ($\alpha = 0.05$, $p < 0.003$) are marked with two asterisks (**). Population abbreviations and sources of data are as listed in Table 3.8.

	FLIX	**NES**	**OUK**	**P+G**	**MS**	**WNAP**	**WNAC**
FLIX		0.127**	0.088**	0.106**	0.168**	0.117**	0.472**
NES	1.835**		0.081**	0.236**	0.182**	0.189**	0.452**
OUK	1.932*	0.748**		0.117**	0.151**	0.148**	0.433**
P+G	0.727**	3.271**	2.538**		0.088**	0.032*	0.387**
MS	1.974**	2.058**	2.028**	1.406**		0.024	0.323**
WNAP	0.902**	2.700**	2.748**	0.379*	0.750		0.292**
WNAC	4.362**	6.621**	6.309**	4.212**	5.286**	3.329**	

Strikingly, 30 of 38 (78.9%) Flixborough samples were assigned with a probability higher than 0.900 to belong to FLIX, and 24 of the 38 individuals (63.2%) were assigned to FLIX with a probability of 1.000. Modern samples rarely showed such strong assignment probabilities, as a value of 1.000 was only seen six times in all other populations. Overall, the majority of modern individuals showed the highest probability of assignment to the putative population that they were placed in for this study and quite low identity to other populations. Modern UK individuals, however, frequently demonstrated (in 26 of 63 individuals) probabilities greater than 0.750 of belonging to more than one of the UK populations of FLIX, NES, and OUK.

III.4.3 Correlation of Genetic and Geographic Distances

The correlation between genetic and geographic distances according to microsatellite data was tested by the completion of Mantel tests of linear regression for plots of each of the calculated microsatellite genetic distances against geographic distances over water between each population. Geographic distances from NES and FLIX to OUK were calculated as weighted averages since samples from OUK were spread over a fairly broad range. Mantel tests using all three genetic distances showed low levels of correlation, two of which are significant at the $p < 0.05$ level (F_{ST}, $r^2 = 0.098$, $p = 0.137$; Rho_{ST}, $r^2 = 0.157$, $p = 0.037$; $(\delta\mu)^2$, $r^2 = 0.157$, $p = 0.017$). Plots of the geographic distances against the residuals for Rho_{ST} and $(\delta\mu)^2$ showed evidence of nonconstant error variance that increased as the geographic separation between samples became larger. These results and the probable relation of this trend to the WNAP population, which is geographically quite far removed from the European populations studied and quite close to WNAC despite showing opposite trends in genetic distances, led to the removal of this

population to better test the trends around the European populations. Correlations with all three genetic distances became much higher and were statistically significant with the removal of WNAP from the analysis (F_{ST}, $r^2 = 0.509$, $p = 0.008$; Rho_{ST}, $r^2 = 0.865$, $p = 0.001$; $(\delta\mu)^2$, $r^2 = 0.763$, $p = 0.018$).

Finally, mantel tests were run on the linear regression of all three microsatellite genetic differentiation measures and geographic distances using only the data from modern day UK populations and Flixborough. This was done to test the significance of the noted discrepancy between the geographic and genetic distances between FLIX, NES and OUK. Correlations of these distances using only these three putative populations were not significant (F_{ST}, $r^2 = 0.209$, $p = 0.699$; Rho_{ST}, $r^2 = 0.860$, $p = 0.835$; $(\delta\mu)^2$, $r^2 = 0.043$, $p = 0.549$).

III.5 Phylogenetic Analysis

Neighbor-joining and maximum parsimony trees were produced to study phylogenetic relationships among the 73 mtDNA haplotypes. The neighbor-joining tree, drawn using the Kimura 2-parameter genetic distance model, is presented in Figure 3.5. Most notable is the polytomy over the large majority of the tree, showing a lack of ability to resolve relationships from this data. The most well defined branch is the clustering of all CHA sequences. One other cluster, supported by just 55% of trees, is interesting for showing evolutionary ties between several populations including OUK, GAL, MS, BS, WNAP and AT. Quite a few dichotomous branches also occur, mostly indicating evolutionary closeness between haplotypes found in the same population. The maximum parsimony tree is presented in Figure 3.6. This tree exhibits an even higher degree of polytomy than the neighbor-joining diagram; however, the cluster of all CHA sequences is

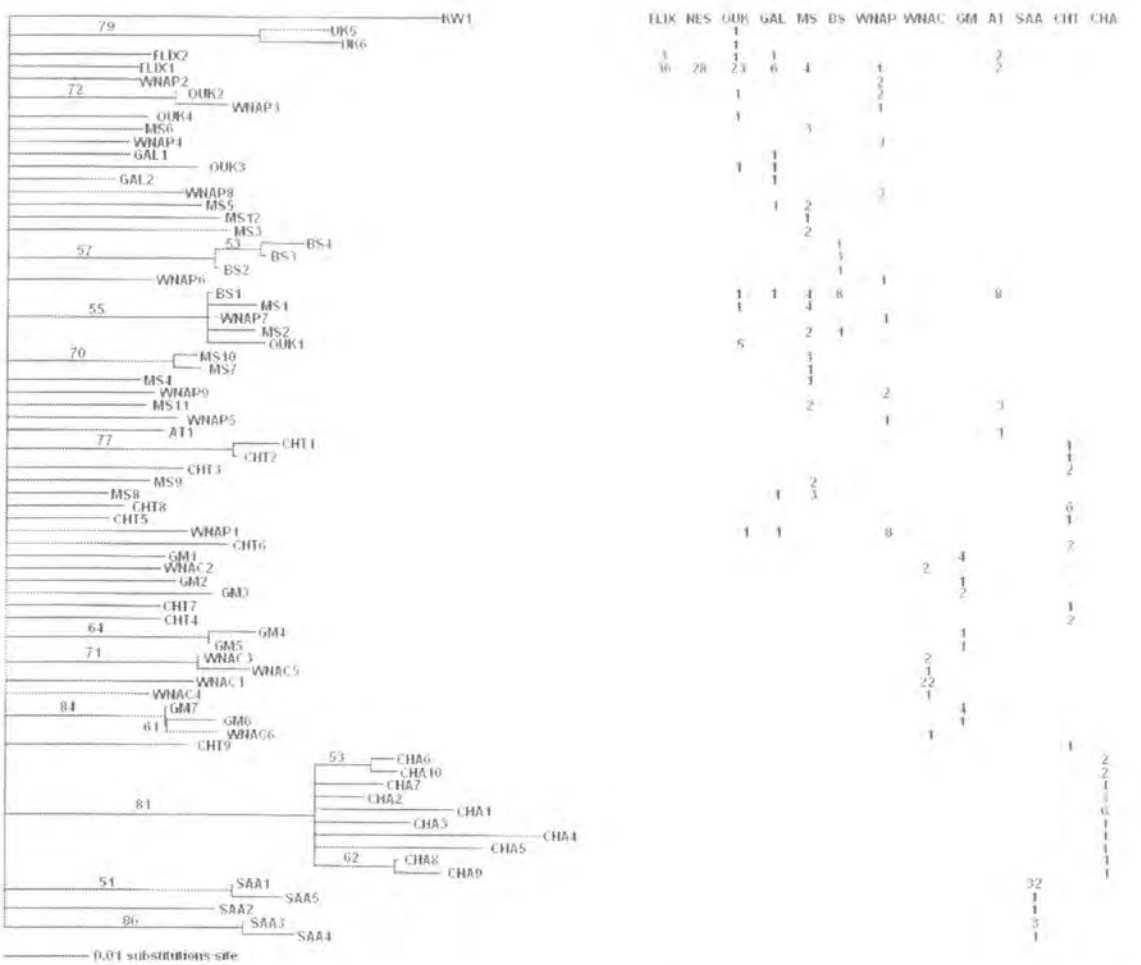

Figure 3.5. Neighbor-joining tree based on a Kimura 2-parameter distance matrix. To the right is listed the number of each haplotype from every population. Bootstrap values of each branch are given above the line and a scale for branches is given in the bottom left corner. Population abbreviations and sources for previously published data are as in Table 3.7. KW1 is a killer whale (*Orcinus orca*) sequence from Natoli et al. (2004) used as the outgroup.

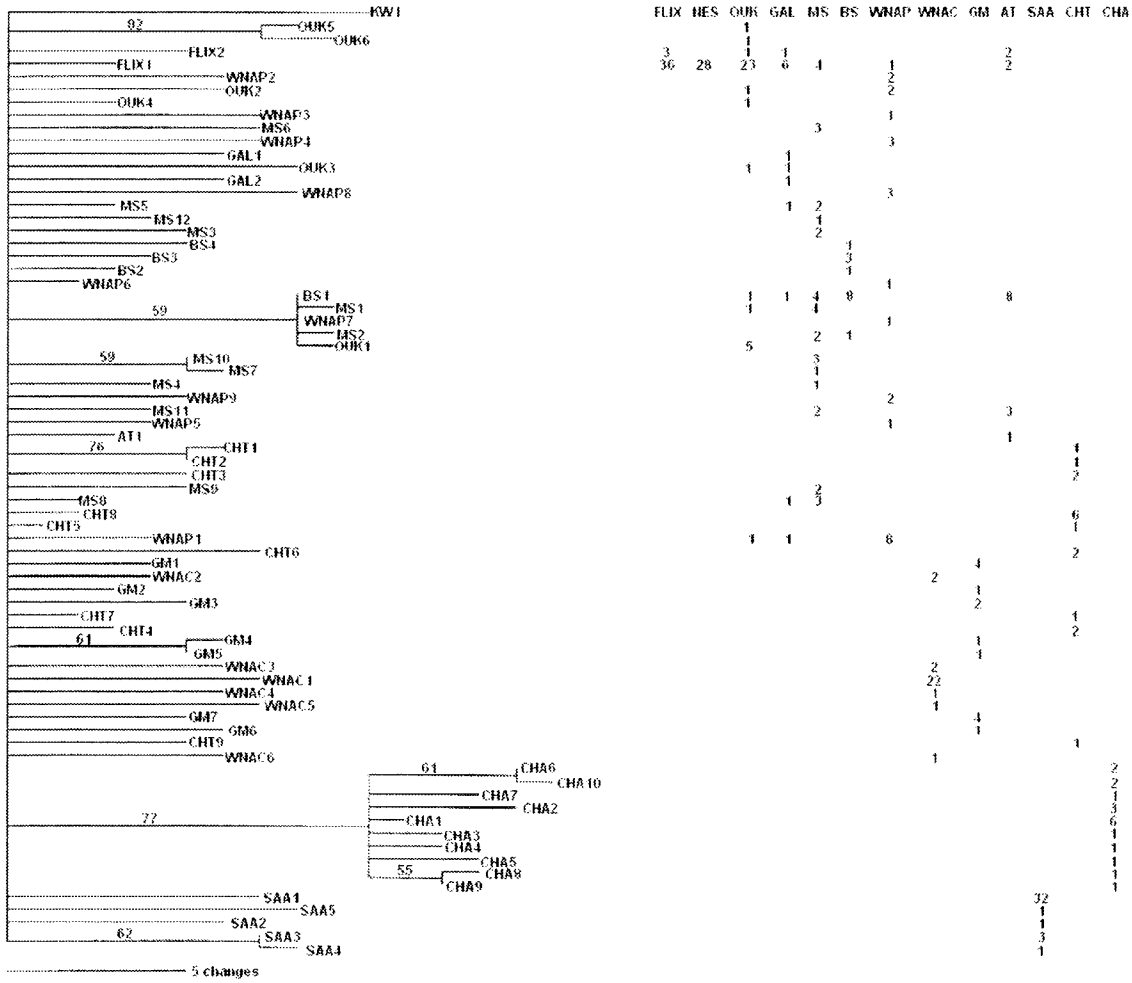

Figure 3.6. Maximum parsimony tree of individual mtDNA haplotypes. Numbers of individuals exhibiting the haplotype from each population are listed to the right of the tree. Bootstrap values are above branches and a scale is presented in the bottom left corner. Population abbreviations and sources for previously published sequences are the same as in Table 3.7. KW1 is a killer whale (*Orcinus orca*) sequence from Natoli et al. (2004) that was used as the outgroup.

still the most well defined grouping and the same cluster containing sequences from OUK, GAL, MS, BS, WNAP and AT exists with a similar level of bootstrap support (59% of trees).

Chapter IV - Discussion

IV.1 Ancient DNA

IV.1.1 Protocol optimization

The fairly large and growing body of published literature involving aDNA research means that much about the optimization of extraction procedures for this type of work can be learned and applied prior to the start of any new study. For this project, two protocols whose basic steps had been published and demonstrated to be successful, were tested with slight modifications made to fit available laboratory conditions. The QIAQuick PCR Purification Kit™ based protocol (Yang et al. 1998) was found to successfully extract DNA from the Flixborough samples and was preferred for its ease and use of less hazardous chemicals. Fairly low initial amplification success, however, did lead to some titration of the procedure. It is evident from the results that a sufficient volume of digest buffer, for the decalcification and cleaning of bone material, as well as a high concentration of proteinase K, for digestion of proteins, are important in achieving successful extraction of aDNA from bones.

The amount of bone powder available for a sample did not significantly affect the ability to amplify mtDNA from a sample, suggesting that ample DNA for mtDNA studies can be obtained from small amounts of bone powder. The need for only a small amount of bone powder is supported by Matisoo-Smith et al. (1997). However, it should be noted that drilling of the Flixborough specimens was not undertaken unless it was thought possible to obtain a volume of approximately 0.2 mL of powder.

The PCR protocol also required little optimization after the start of this study, as previously published guidelines gave initial success. However, the attempted use of lower volumes of aDNA extracts for amplification proved to be less productive. Although lower

amounts of extract can be used to avoid the high concentrations of inhibitors often present in aDNA extracts, amplification efficiencies did not improve in this study with a decreased volume of extract and allelic dropout rates appeared to increase. It therefore seems that high inhibitor concentrations were not the limiting factor in amplification of microsatellites in the Flixborough samples. It could be suggested from this that the extraction protocol used successfully excluded most inhibitors. This is supported by the fact that even in most unsuccessful amplifications primer-dimers could be seen on agarose gels, indicating that the polymerase was active.

IV.1.2 Mitochondrial DNA Sequencing

Because of the high cellular copy number of mtDNA molecules, mtDNA sequence analysis is the most commonly used tool for studies of aDNA. The success rate reported here for amplification of mtDNA PCR products from bone samples, 69.1%, supports the feasibility of this type of analysis. Published studies of aDNA for samples of a similar age and type to those at Flixborough have reported mtDNA amplification success rates as high as > 90% from vertebrate bone and tooth remains (Matisoo-Smith et al. 1997). Barnes et al. (2000), however, reported only a 19.0% success rate for amplification of a similarly sized mtDNA product from goose specimens recovered from Flixborough. Rates of success would be expected to vary greatly among studies though, as factors such as preservation and storage conditions greatly influence the survivability of aDNA (reviewed in MacHugh et al. 2000). The difference in mtDNA success rates between this study and Barnes et al.'s (2000) study of Flixborough specimens therefore is not surprising because Barnes et al. (2000) noted the variability in preservation states, as judged by histological examination, for bones excavated from the Flixborough site.

The frequent problems of ambiguous base positions and even entire regions of hard to read sequences in the mtDNA results were expected for direct sequencing of aDNA because of the inevitably damaged DNA available. Ambiguous base positions in sequences may have resulted from alterations of the DNA molecule over time. Hydrolytic deaminations of cytosine to thymine and other miscoding lesions can yield unclear base positions in direct sequences of aDNA PCR products as some damaged molecules may be amplified along with those still carrying the correct sequence information and direct sequencing will detect both types of products. Paabo et al. (1989, 1990) suggested that numbers of damaged molecules in a sample would likely be too small to be detected in direct sequencing. Nevertheless, if one damaged molecule among a small population of molecules is amplified in an initial round of PCR, it could be amplified to large enough numbers to be detected in the sequence product (Paabo et al. 1990). Of course, difficult base positions may also have been caused by sporadic contaminating sequences present in the extract that amplified with the endogenous DNA, producing overlapping sequences. Unreadable sequences, where many bases could not be determined, may have been due to unspecific binding of the primers during PCR or to jumping between templates during amplification as described by Paabo et al. (1990). The occurrence of these difficulties, however, does not detract from the study because careful editing and replication were applied in obtaining the sequences used for analysis. In fact, these obstacles actually increase the credibility of this study as indicators that the sequences originated from damaged aDNA molecules.

IV.1.3 Microsatellite DNA

The suitability of microsatellite loci for the study of ancient populations is much more debatable. Because microsatellites are located on single-copy nuclear chromosomes

their amplification is much more difficult from aDNA and their interpretation more uncertain. The data obtained in this study illustrated the challenges of working with these loci, from the unclear Genotyper™ peaks to the problem of allelic dropout. Further concern over the use of microsatellite markers comes from the presence of false alleles, which were also seen in this study. False alleles may not only be due to contamination, but may possibly arise from polymerase slippage during early cycles of the PCR reaction (Taberlet et al. 1996) or the formation of recombinant molecules by annealing of partially degraded repeat sequences (Ramos et al. 1995). However, the use of replication to verify difficult peaks, as well as consideration of the various published guidelines (see Gagneux et al. 1997; Miller et al. 2002; Taberlet et al. 1996) based on the multiple-tubes approach (Navidi et al. 1992) for acceptance of genotypes where allelic dropout and false alleles are a concern, allows the use of these loci as credible markers for the study of aDNA, as reported for several studies (e.g. Kurosaki et al. 1993; Nielsen et al. 1999a; Zierdt et al. 1996).

IV.1.4 Ancient DNA Conclusions

This investigation represents the successful use of aDNA techniques for the study of archaeological materials, and the preservation state of the samples is important to the success of any aDNA project. The burial conditions of the Flixborough specimens were suggested to be favorable for preservation of many artifacts due to the neutral soil conditions produced by the presence of alkaline wood ash within the acidic sand of the promonotory upon which the settlement was built (Humber Archaeology Partnership/English Heritage). These conditions of neutral pH were also likely conducive to the survival of DNA within the biotic artifacts (Thomas and Paabo 1993) and the

successful retrieval of DNA from the dolphins investigated in this project and the geese studied by Barnes et al. (2000).

There was concern at the outset of the study that cetacean bones would be difficult to amplify aDNA from because of their porous nature. Picher and Baker (2000) had previously demonstrated the ability to obtain DNA from Hector's dolphin bones up to 130 years old, and the success of this study indicates that even 1,500 year old cetacean bones are suitable for aDNA studies.

The results of this study allow discussion of characteristics of samples that may play a role in DNA survival in ancient specimens. The success in amplification of micosatellites varied greatly over individual samples, indicating that the quality of DNA preservation and the amount of PCR inhibitors present varied significantly amongst the specimens. This is not surprising because preservation of the bones, as assessed visually, varied greatly between individual specimens used in this study, inconsiderate of bone type, and the physical condition of bones has been closely linked to their ability to yield aDNA (Barnes et al. 2000; reviewed in Machugh et al. 2000).

Significant differences in the ability to obtain aDNA from different bone types was not supported by extraction success rates, but significant variance was seen in the amplification of microsatellite loci. It should be noted though that very small sample sizes for some sample types makes the chi-squared test less informative. Previous studies have not specifically looked at bone type but have focused primarily on the preservation state of specimens. The preferential use of teeth is suggested in several studies (for examples see Ginther et al. 1992; Merriwether et al. 1994; Zierdt et al. 1996), though it is not supported here. Teeth actually performed significantly worse in DNA extractions and microsatellite amplifications in this study, but this may be due to the fragile state of teeth obtained from

Flixborough and the inability to obtain sufficient amounts of material for extraction without destroying the sample.

This study also demonstrated significant differences in the ability to amplify varying microsatellite loci in aDNA samples, and suggested a correlation of this success rate with the average size of amplification product for a locus. Nielsen et al. (1999b) noted the importance of using small microsatellite loci in aDNA and all loci used in this study had PCR product sizes of less than 135 bp, well below the 200-250 bp cutoff suggested by Nielsen et al. (1999b). Another concern that differs among loci is the suggestion by Ramos et al. (1995) that dinucleotide repeats are more likely to form chimeric molecules due to annealing of partially degraded repeat segments. This will lead to the observance of false alleles. The occurrence of false alleles only in dinucleotide repeat loci in this study supports this statement. Ramos et al. (1995) considers this as evidence for the unsuitability of dinucleotide markers for aDNA studies, but does note the ability to obtain meaningful estimates of haplotype frequencies from these loci. Here, the relatively small number of false alleles that were observed, 26, over the hundreds of genotypes read suggests that although this phenomenon may occur, it does not discount the ability to obtain meaningful results, as the large majority of genotypes were replicated a minimum of two, and often multiple, times without observance of more than two alleles.

IV.2 Population Analyses

IV.2.1 Intrapopulation Variation

Both the mtDNA and microsatellite markers used in this study consistently showed low levels of genetic variation within the samples from Flixborough. For the mtDNA fragment sequenced, the Flixborough population gave only two haplotypes and the level of

nucleotide diversity was an order of magnitude lower than those of all populations except NES and SAA. For the microsatellite loci studied, FLIX generally showed the lowest values for allele number, allelic richness, and heterozygosity.

Heterozygosity values for FLIX could have been affected by allelic dropout, which is common in aDNA work and known to have occurred in this study. However, allele number and allelic richness should not be affected by this phenomenon unless certain alleles preferentially dropped out. To avoid biased dropout, Nielsen et al. (1999b) suggested the use of microsatellite loci with small ranges in allele size. In this study, all five loci had allele size ranges of less than 35 bp, and three showed ranges of less than 25 bp. Larger allele sizes were commonly detected in loci D22, D08, and D18 within the Flixborough sample, suggesting that smaller allele sizes were not preferentially amplified, although this would be the expected trend for biased dropout. However, homozygous genotypes were predominantly of smaller allele sizes in TtruAAT$_{44}$, MK8, and D18, advancing the idea that the homozygotes reported could possibly have been heterozygotes in which the larger allele had dropped out. Furthermore, the difference in the largest and smallest allele size ranged on average over all loci by 13.2 bp in FLIX and by 26.4 bp in all populations combined. Yet, often the smallest alleles found in other populations were not found in FLIX, suggesting that the low allele number in the Flixborough population is not entirely due to dropout of longer alleles. A study of allelic dropout in samples with low DNA quantity by Gagneux et al. (1997) found that there was no difference in the probability of allelic dropout between longer and shorter alleles. However, Gagneux et al. (1997) used modern shed hair samples where the DNA was likely of a fairly high quality. The fragmented state of aDNA molecules makes dropout of large alleles a more probable occurrence for archaeological samples.

Also of note for Flixborough is the lack of a private haplotype in the mtDNA fragment studied and of a private allele in any of the microsatellite loci used. This is also the case for NES, the only currently defined modern population on the east coast of the United Kingdom. This may suggest that these populations were begun through a founder event from a neighboring population, as founded populations initially receive all their genetic diversity from the few founders that strayed from another population, and will therefore, initially share all alleles with the source population. The domination of FLIX and NES by one mtDNA haplotype may remain from the common haplotype supplied by the founders.

Further suggestive of a founder event in the creation of FLIX and NES is the lack of genetic variation in both populations in comparison with other modern putative bottlenose dolphin populations. Founded populations are expected to show lower levels of variation because they received all their diversity from only a few founding individuals. Natoli et al. (2004) suggested that several coastal populations of bottlenose dolphins might have originated by founder events from nearby offshore populations based on the consistent low variability in coastal populations and high variation in pelagic groups. In this study, the low variation in microsatellite loci in WNAC, as well as the high variability in WNAP, agrees with the idea of low variation in founded coastal populations and high variability in potential source populations. The low variation and dominance of one haplotype in the mtDNA for WNAC and SA also support this idea, but these results originate from the same sequences used by Natoli et al. (2004) when making that conclusion, and therefore cannot lend further support for it. NES is described as a resident, coastal population by Hammond and Thompson (1991) and Wilson et al. (1997), therefore, the low levels of variation found in this population suggest that it could be a coastal population founded in the manner suggested by Natoli et al. (2004).

IV.2.2 Interpopulation Differentiation

The data presented earlier clearly suggest that the sample from Flixborough represents a very unique population. The results of an assignment test show that nearly all samples from Flixborough are assigned with very high probabilities to the defined Flixborough population, and have low probabilities of belonging to all other populations. This gives evidence that these animals are from one defined population group, rather than being hunted in multiple locations and imported to Flixborough.

Flixborough also demonstrates significant differentiation from all populations by each measure of genetic differentiation considered for both mtDNA and microsatellite markers. The one exception that exists is the insignificant Φ_{ST} value between FLIX and NES. This is likely a result of the lack of variation in both populations and their overwhelming domination by the same mtDNA haplotype. As noted in the Results section, all analyses of interpopulation differentiation based on the mtDNA data should be interpreted with some caution because of the small 171 bp fragment size studied.

The differentiation of FLIX from all other populations gives insights into the likelihood of several possible origins for the Flixborough sample. First, it suggests that this was not a population that has since redistributed to another area, as a close genetic tie to that group would be expected, unless of course, samples for this group in its new location were not a part of this study. Flixborough's uniqueness from the geographically close OUK and NES populations also implies that the loss of a Humber Estuary population was not due to a range contraction of a nearby group, as a closer relationship to at least one of the modern UK populations would be predicted. The differentiation of FLIX from other populations could, however, signify an origin for the population from a group of animals

that is no longer extant. This would suggest a displacement of bottlenose dolphins around Great Britain followed by recolonization by a new group sometime over the last 1,500 years. And alternatively, the genetic distinctness of FlIX could further support the hypothesis of a founding event where a sampling effect created distinct allele frequencies that were maintained through isolation.

The dominance of FLIX mtDNA haplotypes by those most common in OUK and NES suggests that the Flixborough samples are from a fellow "British" population. If FLIX was indeed the result of a founding event, the origination of the founders from OUK or GAL is supported by the mtDNA results as these populations include both haplotypes represented at FLIX. The sample from NES does not include the less common Flixborough haplotype. Microsatellite frequencies also show that FLIX shares all its alleles with OUK and P+G, while it contains at least one distinct allele from all other populations. This again points to a founder from one of these groups. If NES is also the product of a founding event, the microsatellite data again points to OUK and P+G as the likely source populations as NES shares all but one allele with OUK and all but two with P+G.

A consistent grouping of populations from the Mediterranean Sea, the Black Sea, Portugal and Galicia, and pelagic West North Atlantic samples was found with all genetic distance measures considered. Additionally, the microsatellite data indicate that the modern British and Flixborough populations are not far removed from this cluster, in comparison to WNAC. The relationship amongst most of these populations is quite reasonable considering their geographic proximity, but the inclusion of the pelagic West North Atlantic samples in this cluster is surprising due to their location on the other side of the Atlantic Ocean. Mantel tests confirmed that the relationship of WNAP to European populations varies significantly from that expected due to geographic separation. The

mtDNA analysis also shows a close relationship of all these populations to *truncatus* type animals off of Africa. Perhaps genetic exchange has always occurred between these groups through long-distance movements, or perhaps today's populations in and just off of the Atlantic originated from one population group which spread to inhabit coastal areas on both sides of the ocean after the release of waters following the last glaciation, as suggested by Natoli et al. (2004). Hewitt (2000) recognized that the genetic consequences of these post-glacial expansions are indeed still expected to be evident. This expansion could have created a series of populations all still linked by their founders from one population group, and the Flixborough founding may have, at some point, been a radiation of this.

IV.2.3 Phylogenetic Analysis

The evolutionary relationships between most mtDNA haplotypes were not defined by the neighbor-joining and maximum parsimony trees of mtDNA sequences. Little resolution was achieved by either method, likely because of the length of the DNA segment used, as better definition for these samples has been found using longer mtDNA sequences in Natoli et al. (2004). The only strongly supported large cluster existing in the trees presented here is the grouping of all CHA haplotypes, supporting the distinction of these animals from the parapatric CHT sample as suggested by Wang et al. (1999). Phylogenetic trees of mtDNA haplotypes also indicated a more weakly supported evolutionary connection for some haplotypes from WNAP, BS, MS and OUK. Possible explanations for the evolutionary link between these populations are presented in the preceding section in the discussion of a possible radiation after the last glaciation.

IV.3 Conclusions

The ultimate goal of this study was to determine the origin of an apparent population of bottlenose dolphins from Anglo-Saxon times, in a location where this species is now rarely seen. The lack of a close association of the Flixborough sample with any other population puts doubt on the hypothesis that Flixborough was part of a population that has moved or contracted since Anglo-Saxon times. The differentiation could be present because the source population for Flixborough is no longer extant, but we would expect to see novel alleles for Flixborough in this case and not such strong mtDNA ties to modern UK populations. Flixborough also could have been part of a population that was not included in this study, though again novel alleles would be expected. Therefore, it seems most likely that the differentiation of Flixborough resulted from a founding event. This is supported by the sample's low genetic variation and sharing of all alleles with a nearby population, OUK. The final hypothesis put forth, that the animals found at the site were hunted elsewhere and transported to Flixborough does not seem likely because Flixborough is not closely tied to any one population that may have been hunted, nor is a mixture of genotypes from other populations indicated.

So, it is suggested here that there was a local, genetically distinct, population of bottlenose dolphins in the Humber Estuary during Anglo-Saxon times, and the numbers of animals found at Flixborough indicate that it was of significant size. Stranding and sighting records throughout the 20th century imply that no large population has existed in the Humber Estuary, or elsewhere on the southern North Sea coast of the United Kingdom, during at least the last 100 years. It therefore seems that the extinction of the Humber Estuary population occurred before the start of this century.

The conclusion of Herman and Dobney (unpublished) that the population of bottlenose dolphins in the Humber Estuary during Anglo-Saxon times was hunted to extinction is therefore a possible scenario. A local coastal population would be quite accessible for hunting from Flixborough due to the settlements' location along the River Trent. The evidence presented by Herman and Dobney (unpublished) for this occurrence, such as the lack of other commonly stranded cetacean species and bottlenose dolphin calves in the site assemblage and the apparent increase in intensity of exploitation over occupation of the site, yield credibility to this hypothesis. It may also be important to note though, that the area around the Humber Estuary passed into Viking control around the 10th century (Loveluck 1997). The Vikings were well known for their sea-faring skills and perhaps could have contributed to the extinction of this population by excessive hunting.

Alternatively, it could be postulated that the extinction of the population was due to environmental changes in the waters off the eastern coast of England over the last 1,000 years. Anderson and Piatt (1999) reported short-term ocean climate shifts that affected marine mammals through changes in prey availability, but not to the extent of extinction or complete abandonment of an area. Hewitt (2000) reviews the redistribution and colonization of areas by many species after glaciation events and the resulting genetic trails remaining in modern animals. However, no such extreme climate changes have occurred over the last 1,000 years. Furthermore, a change in the environment would have likely spurred the Humber Estuary population to redistribute to another area rather than leading to extinction. The wide distribution of bottlenose dolphins throughout the world and the species' often catholic feeding habits (Blanco et al. 2001; Cockroft and Ross 1990; Santos et al. 2001) suggest that these animals likely could have survived in a different area; however, a redistribution is not indicated from the results.

If a local Humber Estuary population of bottlenose dolphins did become extinct as suggested by the results of this study, the loss of a unique group of animals has implications for the modern populations of bottlenose dolphins around Britain, which also occur mostly in small, local populations (Evans 1980). The results of this study indicate that genetic exchange does occur among most groups, especially on the south and west coasts of the British Isles. Still, the lack of variation in the population from northeast Scotland is alarming and suggests possible limited geneflow among local populations. This isolation of the Moray Firth population group also was supported by Parsons et al.'s (2002) study of mtDNA in UK populations. Thompson et al. (2000) used power analysis and population viability analysis models to highlight the vulnerability of the northeast Scotland population and suggested the need for precautionary measures for its conservation. The loss of the Flixborough population warns of the potential for extinction of these small population groups, and can also be used to argue for their protection from human influences. This is especially important in light of the suggestion that numbers of bottlenose dolphins have been decreasing around the British Isles in recent years (Evans 1980; Kayes 1985; Simmonds 1994).

The loss of a bottlenose dolphin population such as Flixborough is also of concern because these animals have been shown to exhibit locally distinct and apparently learned behaviors such as feeding strategies used to exploit locally available food sources (reviewed in Shane 1990), and these behaviors would likely be lost permanently with a local population extinction. A study of the diet of the Flixborough animals, which can be determined by stable isotope analysis and is being completed by another group, will indicate whether these bottlenose dolphins were feeding on local, inshore species of the Humber Estuary. This will help suggest whether such a 'culture' for exploiting local food sources may have been lost. The identification of FLIX as an inshore feeding group would

also lend support to the hypothesis that they were a coastal population group, perhaps founded by a pelagic population, as suggested for several modern coastal populations by Natoli et al. (2004).

Previous studies had already used the comparison of historic and modern DNA from a species for examination of trends in genetic variation and population structure (Nielsen et al. 1999a; Pichler and Baker 2000) and the investigation of population founding events (Hardy et al. 1994). This study confirms the usefulness of aDNA methods for elucidation of significant historical events for a species where previous records and archaeological evidence require further information for clear interpretation, and has yielded valuable insight into the population dynamics of bottlenose dolphins around the United Kingdom and Ireland.

Appendix A

Flixborough Cetacean Remains

Table A.1. Details for all cetacean remains uncovered at the Flixborough site. Species identifications followed by an asterisk were uncertain. All information listed here was obtained from Jerry Herman (NMS). (Table 1 is continued on the next 5 pages.)

Sample Number	Phase	Context	Species I.D.	Age Category	Size	Bone Identification
53	2-3a	4487	*T. truncatus**	Subadult	300	Left rib c.9 fragment
52a	2-3a	4621	*T. truncatus**	Subadult	300	Lumbar vertebra c.15 fragment
52b	2-3a	4621	*T. truncatus**	Subadult	300	Left rib c.10 fragment
23a	2-3a	4963	*T. truncatus*	Subadult	310	Atlas/axis vertebra (fused) fragment
23b	2-3a	4963	*T. truncatus*	Juvenile	250	Tooth
23c	2-3a	4963	*T. truncatus*	Juvenile	250	Right parietal fragment
23d	2-3a	4963	Unid. Cetacean			Cetacean bone fragment
21a	2-3a	5314	*T. truncatus*	Subadult		Tooth
21b	2-3a	5314	*T. truncatus*	Subadult	300	Right rib 12, proximal fragment
19a	2-3a	5369	*T. truncatus*	Adult		Tooth
19b	2-3a	5369	*T. truncatus*	Adult	300	Left parietal fragment
30	3b	3600	*T. truncatus*	Subadult	300	Left scapula fragment
27	3b	4322	*T. truncatus*	Subadult	300	Caudal vertebra c.5 fragment
50a	3b	4323	*T. truncatus*	Adult		Tooth
50b	3b	4323	*T. truncatus*	Juvenile	250	Caudal vertebra c.2 fragment
50c	3b	4323	*T. truncatus**	Juvenile	250	Thoracic vertebra c.3 fragment
50d	3b	4323	*T. truncatus**	Juvenile	250	Left scapula fragment
50e	3b	4323	*T. truncatus**			Cranial fragment, probably frontal or parietal at suture
45a	3b	5617	*T. truncatus*	Subadult	300	Cranial fragment

Sample Number	Phase	Context	Species I.D.	Age Category	Size	Bone Identification
45b	3b	5617	*T. truncatus*	Subadult		Tooth
45c	3b	5617	*T. truncatus**	Adult	300	Lumbar vertebra c.5 frgament
45d	3b	5617	*T. truncatus**	Subadult	300	Left rib 2 fragment
45e	3b	5617	*T. truncatus**	Subadult	300	Rib c.7 proximal fragment
45f	3b	5617	Unid.			Small bone
45g	3b	5617	Unid.			Bone fragment
55	3b	5653	*T. truncatus**	Subadult	300	Left rib c.10 fragment
10	3b	5983	*T. truncatus*	Subadult	275	Posterior part rostrum (end toothrow)
41	3b	6028	*T. truncatus*	Adult	320	Thoracic vertebra 1 fragment
38	3b	6136	*T. truncatus*	Juvenile	250	Lumbar vertebra c.12 fragment
44a	3b	6235	*T. truncatus*	Subadult	275	Rostrum fragment (mid toothrow)
44b	3b	6235	*T. truncatus*	Subadult	275	Cranium fragment, frontal/maxilla/lacrimal (joined) right side
44c	3b	6235	Unid.			Cranial fragment
44d	3b	6235	*T. truncatus*	Adult	330	Atlas/axis, fused, neural spine
44e	3b	6235	*T. truncatus*	Subadult	275	Thoracic vertebra 1 fragment
44f	3b	6235	Unid.			Bone fragment
44g	3b	6235	*T. truncatus*	Adult	330	Lumbar vertebra c.5 fragment
44h	3b	6235	*T. truncatus*	Adult	330	Lumbar vertebra c.6 fragment
44i	3b	6235	*T. truncatus*	Adult	330	Lumbar vertebra c.14 fragment
44j	3b	6235	*T. truncatus*	Subadult	275	Caudal vertebra c.2 fragment
44k	3b	6235	*T. truncatus**	Subadult	275	Thoracic vertebra c.4 small fragment
44l	3b	6235	*T. truncatus*	Adult	330	Right rib 3 proximal fragment
44m	3b	6235	*T. truncatus*	Adult	330	Right rib c.7 proximal fragment
44n	3b	6235	*T. truncatus**	Subadult	275	Right rib c.6 proximal fragment
44o	3b	6235	*T. truncatus**	Subadult	275	Left rib c.1 fragment
44p	3b	6235	*T. truncatus*	Subadult	275	Left rib c.8 proximal fragment
44q	3b	6235	*T. truncatus**	Subadult	275	Right rib 2 proximal fragment
44r	3b	6235	*T. truncatus**	Subadult	275	Right rib 3 proximal fragment

Sample Number	Phase	Context	Species I.D.	Age Category	Size	Bone Identification
47	3b	6441	*T. truncatus**	Juvenile	250	Left rib c.9 fragment
51	3b	7687	*T. truncatus**	Subadult	300	Left rib c.10 fragment
46	3b	8200	*T. truncatus**	Adult	330	Left rib 2 proximal fragment
54	4-5b	1662	*T. truncatus**	Subadult	300	Left rib c.4 proximal fragment
58	4-5b	2720	*T. truncatus**	Subadult/adult	c.320	Sternal rib fragment
31	4-5b	3219	*T. truncatus*	Subadult	300	Left periotic bone (earbone)
36	4-5b	3543	*T. truncatus**	Subadult	300	Lumbar vertebra c.10 fragment
2a	4-5b	3758	*T. truncatus*	Adult	320	Cervical vertebra c.5
2b	4-5b	3758	*T. truncatus*	Juvennile	275	Thoracic vertebra c.10 fragment
2c	4-5b	3758	*T. truncatus*	Juvenile	275	Caudal vertebra c.1 fragment
2d	4-5b	3758	*T. truncatu*s*	Subadult/adult	310	Right rib c.9 fragment
14	4-5b	4195	Unid.			Bone fragment
5a	4-5b	5193	*T. truncatus*	Subadult	275	Thoracic vertebra c.12 fragment
5b	4-5b	5193	*T. truncatus**	Adult	320	Left rib c.9 medial fragment
20a	4-5b	5252	*T. truncatus*	Adult	320	Right dentary fragment (mid toothrow)
20b	4-5b	5252	*T. truncatus**			2 associated cranial fragments
12a	4-5b	5503	*B. acutorostrata*	Juvenile	450	Right squamosal fragment
12b	4-5b	5503	Unid.			Bone fragment
12c	4-5b	5503	Unid.			Bone fragment
12d	4-5b	5503	*T. truncatus*	Subadult	300	Cranial fragment, maxilla, left side
12e	4-5b	5503	*T. truncatus*	Subadult	300	Lumbar vertebra c.12 fragment
42	4-5b	5553	*T. truncatus*	Subadult	300	Caudal vertebra c.1 fragment
18	4-5b	5827	*T. truncatus*	Adult		Tooth
56	4-5b	5968	*B. acutorostrata**	Juvenile	450	Left rib c.4 fragment
4	4-5b	6885	*T. truncatus*	Adult	330	Caudal vertebra c.6 fragment
35	4-5b	8764	*T. truncatus**	Subadult	275	Thoracic vertebra c.6 fragment
6a	4-5b	12057	*T. truncatus*	Adult	300	Anterior part rostrum, premaxilla
6b	4-5b	12057	*T. truncatus*	Adult		Apical tooth

Sample Number	Phase	Context	Species I.D.	Age Category	Size	Bone Identification
6c	4-5b	12057	*T. truncatus**	Adult	300	Vertebral epiphysis fragment, posterior thoracic
6d	4-5b	12057	*T. truncatus*	Adult	300	Thoracic vertebra c.10 fragment
6e	4-5b	12057	*T. truncatus**	Adult	300	Thoracic vertebra c.2 neural spine
6f	4-5b	12057	*T. truncatus**	Adult	300	Left rib c.6 fragment
6g	4-5b	12057	Unid.			Bone fragment
40a	6	1708	*T. truncatus*	Subadult	300	Right rib 1, proximal fragment
40b	6	1708	*T. truncatus*	Subadult	300	Sternum fragment, anterior right side
7	6	1831	*T. truncatus*	Subadult	300	Lumbar vertebra c.10 fragment
25a	6	3236	Unid. *?B. acutor...*			Cetacean bone fragment
25b	6	3236	Unid. *?B. acutor...*			Cetacean bone fragment
11a	6	3610	*G. melas**	Adult	c.550	Lumbar vertebra c.5 fragment
11b	6	3610	*T. truncatus*	Subadult	300	Thoracic vertebra c.10 fragment
11c	6	3610	*T. truncatus*	Subadult	300	Lumbar vertebra c.5 fragment, with 2 associated epiphyses
11d	6	3610	*T. truncatus**	Subadult	300	Left rib 1, distal fragment
11e	6	3610	*T. truncatus**	Subadult	300	Sternal rib fragment
11f	6	3610	*T. truncatus**			Left rib c.13, proximal fragment
1a	6	3891	*T. truncatus*	Adult	300	Anterior part right dentary
1b	6	3891	*T. truncatus*	Adult		8 teeth
1c	6	3891	*T. truncatus*	Subadult	275	Anterior part right dentary
1d	6	3891	*T. truncatus*	Subadult		8 teeth
1e	6	3891	*T. truncatus*	Subadult	275	Thoracic vertebra c.8 fragment
1f	6	3891	*T. truncatus*	Subadult	275	Lumbar vertebra c.5 fragment
1g	6	3891	*T. truncatus*	Subadult	275	Lumbar vertebra c.13 fragment
1h	6	3891	*T. truncatus*	Adult	325	Lumbar vertebra c.9 fragment
1i	6	3891	*T. truncatus*	Adult	300	Lumbar vertebra c.5 fragment
1j	6	3891	*T. truncatus*	Adult	300	Lumbar vertebra c.6 fragment
1k	6	3891	*T. truncatus*	Adult	300	Lumbar vertebra c.7 fragment
9a	6	3891	*T. truncatus*	Subadult	300	Atlas/axis vertebra (fused) fragment

Sample Number	Phase	Context	Species I.D.	Age Category	Size	Bone Identification
9b	6	3891	*T. truncatus*	Subadult	300	Thoracic vertebra c.7 fragment
9c	6	3891	*T. truncatus*	Adult	325	Lumbar vertebra c.14 fragment
9d	6	3891	*T. truncatus*	Adult	325	Lumbar vertebra c.15 fragment
9e	6	3891	*T. truncatus*			Vertebral process fragment
28	6	3891	Unid.			Bone fragment with 3 articulations
43a	6	3891	*T. truncatus*	Adult	325	Maxilla fragment (mid toothrow), right side
43b	6	3891	*T. truncatus**	Subadult		Cranial fragment, parietal
43c	6	3891	Unid.			Cranial fragment
43d	6	3891	Unid.			Cranial fragment
43e	6	3891	Unid.			Cranial fragment
43f	6	3891	Unid.			Cranial fragment
43g	6	3891	*T. truncatus*	Adult	325	Thoracic vertebra 3 fragment, neural spine
43h	6	3891	*T. truncatus*	Adult	325	Thoracid vertebra 1 fragment, neural spine
43i	6	3891	*T. truncatus*	Adult	325	Thoracic vertebra 2 fragment, neural arch left side
43j	6	3891	*T. truncatus*	Subadult	275	Thoracic vertebra 2 fragment
43k	6	3891	*T. truncatus*	Subadult	275	Lumbar vertebra c.15 fragment
43l	6	3891	*T. truncatus*	Subadult	275	Caudal vertebra c.2 fragment
43m	6	3891	*T. truncatus*	Subadult	275	Caudal vertebra c.1 fragment
43n	6	3891	*T. truncatus**	Subadult	300	Left rib 2 fragment
43o	6	3891	*T. truncatus**	Subadult	300	Right rib c.7 fragment
43p	6	3891	*T. truncatus*	Subadult		Vertebral epiphysis fragment, anterior caudal
43q	6	3891	*T. truncatus*	Subadult		Vertebral epiphysis fragment, anterior lumbar
43r	6	3891	Unid. *?B. acutor...*		c.550	Left rib fragment
48a	6	3891	*T. truncatus**	Adult	325	Right rib c.7 fragment
48b	6	3891	*T. truncatus**	Subadult	300	Left rib c.11 fragment
49a	6	3891	*T. truncatus*	Subadult	275	Lumbar vertebra c.6 fragment
49b	6	3891	*T. truncatus**	Subadult	275	Right rib c.4 fragment
49c	6	3891	*T. truncatus**	Subadult	275	Right c.10 fragment

Sample Number	Phase	Context	Species I.D.	Age Category	Size	Bone Identification
61	6	3891	*T. truncatus**	Subadult	275	Right rib 2 proximal fragment
60	6	3891	*T. truncatus**	Subadult	275	Left rib c.9 fragment
8a	6	5871	*T. truncatus*	Adult	325	Lumbar vertebra c.12 fragment
8b	6	5871	*T. truncatus*	Adult	325	Caudal vertebra c.8 fragment
8c	6	5871	*T. truncatus*	Adult	325	Skull fragment, maxilla/frontal, right side
22	6	6046	*T. truncatus*	Adult	320	Rostrum fragment (mid toothrow)
3	6	6498	*T. truncatus*	Subadult	310	Lumbar vertebra c.10 fragment
37	6	10296	*T. truncatus*	Juvenile	250	Caudal vertebra c.4 fragment
57	6	10333	Unid.			Cetacean bone fragment
24a	6iii	636	B. acutorostrata	Juvenile	450	Left ulna fragment
24b	6iii	636	Unid. *?B. acutor...*			Cranial fragment
13a	6iii	779	B. acutorostrata	Juvenile	450	Left maxilla fragment (7 pieces joined)
13b	6iii	779	Unid. *?B. acutor...*			Cetacean small bone fragment
33	6iii	1269	*T. truncatus**	Adult	320	Thoracic vertebra c.2 fragment
59a	6iii	1282	*T. truncatus*	Subadult	275	Cranial fragment, premaxilla/maxilla
59b	6iii	1282	Unid.	Adult		Cranial fragment (2 pieces joined)
34	6iii	1283	*T. truncatus*	Adult	320	Lumbar vertebra c.5 fragment
32	6iii	1457	*T. truncatus*	Adult	320	Thoracic vertebra c.5 fragment
29a	6iii	1459	*T. truncatus*	Subadult		Tooth
29b	6iii	1459	*T. truncatus**	Subadult	300	Thoracic vertebra c.4 fragment
62	6iii	1459	*T. truncatus**	Adult		Tooth
26	6iii	1740	*T. truncatus*	Subadult	300	Maxilla fragment (mid toothrow)
16	6iii	3452	*T. truncatus*	Subadult		Tooth
39	6iii	7054	*T. truncatus*	Adult	320	Caudal vertebra c.7 fragment
17	?	1688	*T. truncatus*	Subadult		Tooth
15	?	2570	*T. truncatus*	Adult		Tooth

Appendix B

Raw Data for Mitochondrial DNA and Microsatellite Results

Table B.1. Details of extraction/mtDNA amplification and sequencing for all Flixborough samples used in this study. For each extraction attempt, the column under "Ext./Amp." lists the volume in mL of digest buffer used in extraction, followed by a + or – to signify whether a mtDNA PCR product was achieved. Those followed by an asterisk gave extremely faint bands for which sequencing was not attempted. Under the "Sequencing" column, the primer (A or B) used for sequencing is given, followed by a + or – to indicate whether this yielded a readable sequence. In the final column, the haplotype, if determined, is given for each sample. Haplotypes 1 and 2 refer to FLIX1 and FLIX2, respectively in Figures 3.5 and 3.6, and in Table 2 of this appendix. (Table 1 is continued on the following 2 pages)

Sample	Extraction #1		Extraction #2		Extraction #3		Extraction #4		Hap
	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	
53	6–								
52a	7–								
52b	4+	A+, B+	7+	A+	2+				2
23a	4+	A+	5–		8+	A+, B+	6+		1
23b	3–								
23c	3–		3+						
23d	8–		7–						
21b	8–		6+	B+	6+	B+, A+	3+		2
19b	3+	A+	3+	B+	2+				1
30	7–		7+	B+	7+	A–, A+, B+	4+		1
27	7–								
50b	7–								
50d	4–								

Sample	Extraction #1		Extraction #2		Extraction #3		Extraction #4		Hap
	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	
50e	6+	B+	3+	B+, A+	3+				1
45c	7+	A+, B+	6+	A+	3+				1
45d	4+	A+	7+	B+	5+				2
55	6–		5–						
10	8–		7–						
41	5+*		8+	B+	6+	B+, A+	7+		1
38	5–		7+	B+	6+	B+, A+	5+		1
44a	4–		6–						
44i	5+*								
44j	8+								
44l	8+	B+	7+	B+, A+	7+				1
47	6–		6+	B+	6+	B+, A+	4+		1
51	7+	A+, B+	6+	B+	4+				1
46	4+	B+, A+	6+	B+	6+				1
54	7–								
58	4+	A+	6+	B+	4+				1
2a	5+	A+	3+	B–, B+	7+				1
2b	7+	A+	8+	B+	8+	B+			1
2d	5+	B–, B+	5+	A–					
5a	8+	A+	8+	B+	8+				1
5b	6+	A+	6+	B+	3+				1
20a	5–								
20b	5+	A+, B+	8+	A+	7+				1
12b	7–								
12d	6+								
12e	8–								
42	6+	A+	6+	B+	7+				1
4	8+	A+	8+	B+	7+				1

Sample	Extraction #1		Extraction #2		Extraction #3		Extraction #4		Hap
	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	Ext./Amp.	Sequencing	
6a	8+	A–, B+	8+	A+, B+	8+				1
6d	7+	A+, B+	8+	B+	6+				1
6e	6+	A+	4+	B+	5+	B–			1
40b	6+	A+	7+	B+	4+				1
7	7+	B+	6+	B+, B–, A+	7+				1
11c	6+	A+, B+	7+	B+	8+				1
11d	5+	B+	4+	B+, A+	3+				1
1b	4+								
1d	5–								
1e	6+	A+	8+	B+	6+				1
1h	6+	A+	6+	B+	6+				1
1j	8+	A+, B+	8+	B+	7–				1
9b	5–		6+	A+	6+	B+	5+		1
8c	6+	A+	3+	B+	6+				1
3	6–		8–						
37	4+	A+	7+	B+	3+				1
33	6–		8+						
34	6+	B–							
32	6+	B–, B+	7+	B+, A+	6+				1
29a	4–								
29b	7–								
26	8+	B–, B–, B+, A+	8+	B+	6+				1
16	5+	B+	4+	B+, A+	3+				1
39	8+	A+	7+	B+	8+				1
17	3–								

Table B.2. mtDNA haplotype frequencies reported as numbers of sequences. Population abbreviations and sources of sequences are as indicated in Table 3.7. (Table 2 is continued on the following two pages.)

Haplotype	FLIX	NES	OUK	GAL	MS	BS	WNAP	WNAC	GM	AT	SAA	CHT	CHA
FLIX1	36	28	23	6	4	0	1	0	0	2	0	0	0
FLIX2	3	0	1	1	0	0	0	0	0	2	0	0	0
OUK1	0	0	5	0	0	0	0	0	0	0	0	0	0
OUK2	0	0	1	0	0	0	2	0	0	0	0	0	0
OUK3	0	0	1	1	0	0	0	0	0	0	0	0	0
OUK4	0	0	1	0	0	0	0	0	0	0	0	0	0
OUK5	0	0	1	0	0	0	0	0	0	0	0	0	0
OUK6	0	0	1	0	0	0	0	0	0	0	0	0	0
GAL1	0	0	0	1	0	0	0	0	0	0	0	0	0
GAL2	0	0	0	1	0	0	0	0	0	0	0	0	0
MS1	0	0	1	0	4	0	0	0	0	0	0	0	0
MS2	0	0	0	0	2	1	0	0	0	0	0	0	0
MS3	0	0	0	0	2	0	0	0	0	0	0	0	0
MS4	0	0	0	0	1	0	0	0	0	0	0	0	0
MS5	0	0	0	1	2	0	0	0	0	0	0	0	0
MS6	0	0	0	0	3	0	0	0	0	0	0	0	0
MS7	0	0	0	0	1	0	0	0	0	0	0	0	0
MS8	0	0	0	1	3	0	0	0	0	0	0	0	0
MS9	0	0	0	0	2	0	0	0	0	0	0	0	0
MS10	0	0	0	0	3	0	0	0	0	0	0	0	0
MS11	0	0	0	0	2	0	0	0	0	3	0	0	0
MS12	0	0	0	0	1	0	0	0	0	0	0	0	0
BS1	0	0	1	1	4	8	0	0	0	8	0	0	0
BS2	0	0	0	0	0	1	0	0	0	0	0	0	0
BS3	0	0	0	0	0	3	0	0	0	0	0	0	0

Haplotype	FLIX	NES	OUK	GAL	MS	BS	WNAP	WNAC	GM	AT	SAA	CHT	CHA
BS4	0	0	0	0	0	1	0	0	0	0	0	0	0
WNAP1	0	0	1	1	0	0	8	0	0	0	0	0	0
WNAP2	0	0	0	0	0	0	2	0	0	0	0	0	0
WNAP3	0	0	0	0	0	0	1	0	0	0	0	0	0
WNAP4	0	0	0	0	0	0	3	0	0	0	0	0	0
WNAP5	0	0	0	0	0	0	1	0	0	0	0	0	0
WNAP6	0	0	0	0	0	0	1	0	0	0	0	0	0
WNAP7	0	0	0	0	0	0	1	0	0	0	0	0	0
WNAP8	0	0	0	0	0	0	3	0	0	0	0	0	0
WNAP9	0	0	0	0	0	0	2	0	0	0	0	0	0
WNAC1	0	0	0	0	0	0	0	22	0	0	0	0	0
WNAC2	0	0	0	0	0	0	0	2	0	0	0	0	0
WNAC3	0	0	0	0	0	0	0	2	0	0	0	0	0
WNAC4	0	0	0	0	0	0	0	1	0	0	0	0	0
WNAC5	0	0	0	0	0	0	0	1	0	0	0	0	0
WNAC6	0	0	0	0	0	0	0	1	0	0	0	0	0
GM1	0	0	0	0	0	0	0	4	0	0	0	0	0
GM2	0	0	0	0	0	0	0	1	0	0	0	0	0
GM3	0	0	0	0	0	0	0	2	0	0	0	0	0
GM4	0	0	0	0	0	0	0	1	0	0	0	0	0
GM5	0	0	0	0	0	0	0	1	0	0	0	0	0
GM6	0	0	0	0	0	0	0	1	0	0	0	0	0
GM7	0	0	0	0	0	0	0	4	0	0	0	0	0
AT1	0	0	0	0	0	0	0	0	0	1	0	0	0
SAA1	0	0	0	0	0	0	0	0	0	0	32	0	0
SAA2	0	0	0	0	0	0	0	0	0	0	1	0	0
SAA3	0	0	0	0	0	0	0	0	0	0	3	0	0
SAA4	0	0	0	0	0	0	0	0	0	0	1	0	0
SAA5	0	0	0	0	0	0	0	0	0	0	1	0	0

Haplotype	FLIX	NES	OUK	GAL	MS	BS	WNAP	WNAC	GM	AT	SAA	CHT	CHA
CHT1	0	0	0	0	0	0	0	0	0	0	0	1	0
CHT2	0	0	0	0	0	0	0	0	0	0	0	1	0
CHT3	0	0	0	0	0	0	0	0	0	0	0	2	0
CHT4	0	0	0	0	0	0	0	0	0	0	0	2	0
CHT5	0	0	0	0	0	0	0	0	0	0	0	1	0
CHT6	0	0	0	0	0	0	0	0	0	0	0	2	0
CHT7	0	0	0	0	0	0	0	0	0	0	0	1	0
CHT8	0	0	0	0	0	0	0	0	0	0	0	6	0
CHT9	0	0	0	0	0	0	0	0	0	0	0	1	0
CHA1	0	0	0	0	0	0	0	0	0	0	0	0	6
CHA2	0	0	0	0	0	0	0	0	0	0	0	0	3
CHA3	0	0	0	0	0	0	0	0	0	0	0	0	1
CHA4	0	0	0	0	0	0	0	0	0	0	0	0	1
CHA5	0	0	0	0	0	0	0	0	0	0	0	0	1
CHA6	0	0	0	0	0	0	0	0	0	0	0	0	2
CHA7	0	0	0	0	0	0	0	0	0	0	0	0	1
CHA8	0	0	0	0	0	0	0	0	0	0	0	0	1
CHA9	0	0	0	0	0	0	0	0	0	0	0	0	1
CHA10	0	0	0	0	0	0	0	0	0	0	0	0	2

Table B.3. Microsatellite allele frequencies for each locus and population reported as proportions. Each table presents allele frequencies for one locus. A) TtruAAT$_{44}$, B) D22, C)D08, D)MK8, E) D18. The number of genotypes obtained, or sample size (n), for each locus is given underneath the population abbreviations in every table. Population abbreviations and sources of data are as reported in Table 3.8. (Table 3 is continued on the following four pages.)

A) TtruAAT$_{44}$

Allele Size	**FLIX** $n = 33$	**NES** $n = 27$	**OUK** $n = 31$	**P+G** $n = 28$	**MS** $n = 29$	**WNAP** $n = 27$	**WNAC** $n = 27$
82	0.000	0.000	0.000	0.000	0.207	0.037	0.019
85	0.015	0.019	0.016	0.018	0.103	0.111	0.000
88	0.045	0.574	0.226	0.036	0.017	0.074	0.556
91	0.621	0.019	0.177	0.304	0.345	0.167	0.407
94	0.091	0.310	0.097	0.125	0.000	0.185	0.000
97	0.227	0.241	0.452	0.304	0.086	0.389	0.019
100	0.000	0.019	0.016	0.107	0.155	0.037	0.000
103	0.000	0.000	0.000	0.036	0.069	0.000	0.000
106	0.000	0.000	0.016	0.071	0.017	0.000	0.000

B) D22

Allele Size	**FLIX** *n* = 22	**NES** *n* = 26	**OUK** *n* = 31	**P+G** *n* = 29	**MS** *n* = 28	**WNAP** *n* = 26	**WNAC** *n* = 27
111	0.000	0.000	0.016	0.000	0.000	0.000	0.000
113	0.068	0.192	0.065	0.172	0.286	0.192	0.000
115	0.000	0.000	0.016	0.017	0.071	0.000	0.000
119	0.000	0.019	0.032	0.000	0.000	0.038	0.000
121	0.682	0.654	0.419	0.259	0.107	0.231	0.093
123	0.000	0.000	0.048	0.034	0.071	0.173	0.037
125	0.136	0.115	0.194	0.276	0.357	0.154	0.111
127	0.000	0.000	0.048	0.121	0.036	0.154	0.315
129	0.114	0.000	0.113	0.000	0.000	0.000	0.111
131	0.000	0.019	0.016	0.034	0.036	0.019	0.000
133	0.000	0.000	0.032	0.086	0.036	0.038	0.315
135	0.000	0.000	0.000	0.000	0.000	0.000	0.019

C) D08

Allele Size	FLIX $n = 20$	NES $n = 27$	OUK $n = 29$	P+G $n = 29$	MS $n = 29$	WNAP $n = 26$	WNAC $n = 27$
93	0.000	0.000	0.000	0.000	0.034	0.000	0.000
95	0.000	0.000	0.000	0.000	0.017	0.038	0.093
101	0.000	0.000	0.000	0.000	0.000	0.019	0.019
103	0.000	0.000	0.000	0.034	0.052	0.135	0.019
105	0.125	0.204	0.017	0.207	0.259	0.135	0.593
107	0.000	0.000	0.103	0.121	0.121	0.269	0.241
109	0.075	0.000	0.017	0.052	0.121	0.115	0.019
111	0.350	0.722	0.741	0.414	0.276	0.192	0.019
113	0.450	0.074	0.034	0.121	0.103	0.019	0.000
115	0.000	0.000	0.034	0.017	0.017	0.038	0.000
117	0.000	0.000	0.017	0.000	0.000	0.019	0.000
119	0.000	0.000	0.000	0.034	0.000	0.000	0.000
121	0.000	0.000	0.000	0.000	0.000	0.019	0.000
123	0.000	0.000	0.034	0.000	0.000	0.000	0.000

D) MK8

Allele Size	FLIX $n = 23$	NES $n = 20$	OUK $n = 29$	P+G $n = 24$	MS $n = 28$	WNAP $n = 22$	WNAC $n = 19$
95	0.000	0.000	0.017	0.021	0.036	0.045	0.000
99	0.000	0.000	0.069	0.125	0.250	0.000	0.000
101	0.000	0.000	0.000	0.021	0.036	0.091	0.000
103	0.000	0.000	0.017	0.125	0.179	0.114	0.026
105	0.326	0.225	0.190	0.313	0.107	0.341	0.421
107	0.283	0.125	0.172	0.208	0.232	0.205	0.000
109	0.043	0.025	0.155	0.093	0.143	0.091	0.026
111	0.348	0.500	0.379	0.125	0.018	0.091	0.316
113	0.000	0.125	0.000	0.000	0.000	0.023	0.158
115	0.000	0.000	0.000	0.000	0.000	0.000	0.053

E) D18

Allele Size	FLIX $n = 29$	NES $n = 27$	OUK $n = 35$	P+G $n = 29$	MS $n = 30$	WNAP $n = 26$	WNAC $n = 27$
72	0.000	0.000	0.000	0.000	0.000	0.019	0.000
74	0.052	0.000	0.057	0.224	0.083	0.115	0.000
76	0.000	0.000	0.043	0.121	0.033	0.096	0.000
80	0.000	0.000	0.029	0.000	0.000	0.058	0.000
82	0.655	0.389	0.157	0.155	0.133	0.077	0.500
84	0.000	0.000	0.000	0.000	0.017	0.019	0.000
86	0.034	0.111	0.114	0.052	0.050	0.096	0.278
88	0.000	0.000	0.014	0.103	0.267	0.212	0.204
90	0.000	0.000	0.000	0.069	0.000	0.115	0.019
92	0.155	0.000	0.014	0.069	0.183	0.115	0.000
94	0.000	0.000	0.029	0.017	0.000	0.038	0.000
96	0.000	0.056	0.029	0.052	0.167	0.000	0.000
98	0.103	0.444	0.482	0.138	0.067	0.038	0.000
100	0.000	0.000	0.014	0.000	0.000	0.000	0.000
106	0.000	0.000	0.014	0.000	0.000	0.000	0.000

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