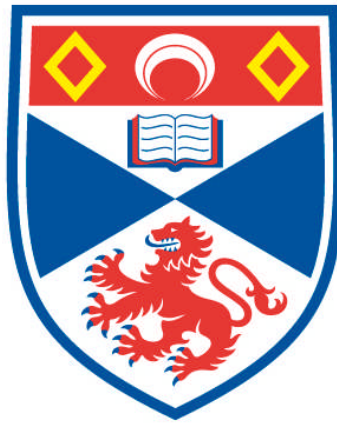


**GENETIC CHARACTERISATION AND SOCIAL STRUCTURE
OF THE EAST SCOTLAND POPULATION OF BOTTLENOSE
DOLPHINS (TURSIOPS TRUNCATUS)**

Valentina Islas-Villanueva

**A Thesis Submitted for the Degree of PhD
at the
University of St Andrews**



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**Genetic Characterisation and Social Structure of
the East Scotland population of bottlenose
dolphins (*Tursiops truncatus*)**

Valentina Islas-Villanueva

Submitted for the degree of Doctor of Philosophy to the University of St
Andrews

September, 2009

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I, Valentina Islas-Villanueva, hereby certify that this thesis, which is approximately 25 000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in October 2005 and as a candidate for the degree of PhD. in October 2006; the higher study for which this is a record was carried out in the University of St Andrews between 2005 and 2009.

date signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

date signature of supervisor

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St Andrews

I love how it comes right out of the blue
North Sea edge, sunstruck with oystercatchers.

A bullseye centred at the outer reaches,
A haar of kirks, one inch in front of beyond.

Robert Crawford

Genetic characterisation and social structure of the Eastern Scotland population of bottlenose dolphins (*Tursiops truncatus*)

Summary

The Eastern Scottish population of bottlenose dolphins (*Tursiops truncatus*) is the northernmost population of this species. The resident core of this population consists of 120 to 150 different individuals. This small size and its geographical isolation from other populations raises questions about its viability and whether the population has behavioural patterns that differ from those common to other populations of the same species. Microsatellite genetic diversity was low and mitochondrial DNA genetic diversity values were lowest in East Scotland compared to other populations worldwide and to neighbouring populations around UK waters. It has been well documented, from four different field sites worldwide, that male bottlenose dolphins form alliances with preferred male associates. These alliances can last for several years and the males involved males show association coefficients similar to those of mothers and calves (0.8-1.0). These alliances appear to be of great importance in obtaining matings for the males. In the Eastern Scottish population males do not form alliances. No evidence of strong associations between individuals of either sex was found and there was no correlation between association and relatedness patterns. I suggest that the isolation and small size of the population together with reduced genetic diversity affects the pressure of kin selection for altruistic behaviours. There is no gain in competing or associating with close relatives for access to mates and it might be more important to avoid inbreeding by dispersing. Although evidence of gene flow between East Scotland and its neighbouring populations was not confirmed with Bayesian clustering analysis, a small set of individuals from Wales were found to be closely related to individuals from the East Coast of Scotland. In general the dynamics found in UK water populations resemble those of the Western North Atlantic with sympatric populations of coastal as well as pelagic individuals.

Chapter 1 Introduction:

1.1. Genetic consequences of social organization

Natural populations are generally structured in subpopulations interconnected by different levels of migration (Perrin & Mazalov 2000). Gene flow is the main force that determines subpopulation structure and how independently they evolve from each other (Slatkin 1987). For gene flow to occur between two populations, they need to overlap in their distribution, while being sexually active and receptive to each other (Slater & Halliday 1994). These actions must be mediated by exchanging signals to attract mates; sometimes mates are chosen to be from the same population and sometimes they are from a distant one (Slater & Halliday 1994).

Individuals can gain 'inclusive fitness' through the reproduction of related individuals as well as through their own reproduction (Hamilton 1963); (Maynard-Smith 1964). This idea supports behaviours such as altruism, aggression, cooperation, selfishness and spite (Griffin & West 2002). If a particular gender is philopatric, individuals of this population will spend more time with their close relatives, which will allow kin selection to operate on social behaviours (Maynard-Smith 1964).

A common pattern found in mammals is male biased dispersal and female philopatry (Greenwood 1980). These patterns reflect a complex decision making process and could be a result of several scenarios. In promiscuous or polygynous species females invest more in breeding, so they have to focus on obtaining resources, while males compete for mates (Perrin & Mazalov 2000). If neither sex dispersed, inbreeding would become more likely. This could result in inbreeding depression with mated individuals being closely related producing offspring with reduced fitness (Saccheri et al. 1996).

In a highly inbred population, females would suffer the costs of inbreeding depression by investing their resources in non-viable offspring. Under this scenario, they would be more likely to choose, when possible, migrant mates, instead of local ones, thus forcing local males to disperse (Lehmann & Perrin 2003). Amos et al. (2001) showed that certain species of marine mammals could avoid inbreeding by selecting mates that are highly dissimilar to themselves. Another possibility could be that as females suffer more in an inbred population they would be expected to disperse (Waser et al. 1986).

These behavioural differences have obvious implications in the population structure of mammals. Maternal stable relationships are important in African elephants (*Loxodonta africana*); they live in fission-fusion groups with core groups of females comprised by first order relatives (Archie et al. 2008). The strong associations of female relatives and male dispersal are also common in rhesus monkeys (*Macacca mulata*) (Melnick 1987; Widdig et al. 2006). On the other hand maternal relatedness does not seem to affect strong female associations in bonobos (*Pan paniscus*) (Hashimoto et al. 1996) or male affiliations in chimpanzees (*Pan troglodytes*) (Goldberg & Wrangham 1997; Mitani et al. 2000). In Baboons the differences in reproductive success between males and their short term dominant state, result in a population that is sub-structured in age groups of paternal relatives (Altmann et al. 1996).

1.2. Social structure in Odontocetes

The order *cetacea* is subdivided into the mystecetes (baleen whales) and the odontocetes (toothed whales, dolphins and porpoises) (Rice 1989a).

Odontocetes show a variety of social arrangements both between and within species and in general they form more complex associations than mystecetes (Connor et al. 2000a). There are a few species of odontocetes that have been widely studied such as killer whales (*Orcinus orca*), sperm whales (*Physeter*

macrocephalus), pilot whales (*Globicephala melas*) and bottlenose dolphins (*Tursiops spp*). All these show a variety of complex patterns of association and relatedness that will be briefly described below.

Killer whales off southwest Canada live in sympatric populations that have been named resident and transient. Resident killer whales feed primarily on fish and live in matrilineal groups where males and females do not disperse, they gather with other matrilineal groups forming pods. Transient killer whales feed on other marine mammals and they also gather in matrilineal groups of small size that require dispersal from the natal group (Baird 2000).

Sperm whales are also grouped in female matrilineal groups of around 10 individuals that are kin related which associate with other groups for a certain amount of time (Richard et al. 1996). Male sperm whales on the other hand leave their natal groups to join 'bachelor' groups. As they grow larger they become more solitary and migrate to higher latitudes (Rice 1989b). Pilot whales (*Globicephala melas*) also associate with kin and they form very stable family bonds. It seems that both mature males and females stay in their natal pods throughout their lives but males only reproduce with females from other pods (Amos et al. 1993).

Bottlenose dolphins (*Tursiops spp*) show a variety of complex social behaviours that will be described in more detail in Chapters 3 and 4. They live in fission-fusion societies (Wells et al. 1980) but despite this characteristic of their societies, long term associations of bottlenose dolphins have been documented in some well studied populations since the 1970s (Scott et al. 1990; Smolker et al. 1992; Wursig & Harris 1990; Wursig & Wursig 1977). Males can show strong bonds of 2 or 3 individuals that compete for access to females (Connor et al. 1992a; Connor et al. 1992b; Connor et al. 2000b; Moller et al. 2001; Parsons et al. 2003; Wells et al. 1987) but they can also be solitary (Wells et al. 1980). Females in some populations show a large number of

associates (Smolker 1992), in others they form female bands of close relatives (Wells et al. 1987) and in others they can be found in groups of similar reproductive state (Möller & Harcourt 2008). Male-female relationships seem to be restricted to mother- calf pairs or to sexual interactions (Connor et al. 2000b).

1.3. The species studied

The bottlenose dolphin *Tursiops truncatus* (Montagu, 1821), is a well known and studied odontocete species. It shows a worldwide distribution and its presence is greater in coastal regions of tropical and temperate waters (Shane 1988), though they also inhabit pelagic habitats (Jefferson et al. 1996) (fig. 1).

Besides its presence in the United Kingdom and the north of Europe, it is almost always found in latitudes between 45° north and south (Jefferson et al. 1996).

In the Atlantic Ocean it occurs in the northern Gulf of Mexico, Georges Bank off Massachusetts, the British Isles, the Baltic Sea including the Gulf of Finland, the Mediterranean and Black seas, Newfoundland and Norway (Rice 1998). Its presence has been well documented down to the southern Gulf of Mexico, the Mexican Caribbean (Delgado-Estrella 2002) and Belize (Bilgre et al. 1995).

In the Pacific the distribution ranges north to the Bo Hai, East China Sea, central Honshu, Kure Atoll, Hawaii, Isla Guadalupe (Rice 1998), the inner Gulf of California (Ballance 1990), Monterey Bay in California to Puget Sound in Washington State. In the Southern Hemisphere it occurs south to Golfo San Matias in Argentina, 18°S in northern Namibia, Port Elizabeth in Cape Province, Walters Shoal in the southwestern Indian Ocean, the southern coast

of Australia including Tasmania, South Island (Rice 1998) and Doubtful Sound (Williams et al. 1993) in New Zealand, and Concepción, Chile (Rice 1998).

Populations all over the species worldwide distribution show different behavioural specializations and different phenotypes. These differences are related to local adaptations or a particular social structure but it is not clear if they reflect real phylogenetic separations or just a great phenotypic plasticity (Curry & Smith 1997).

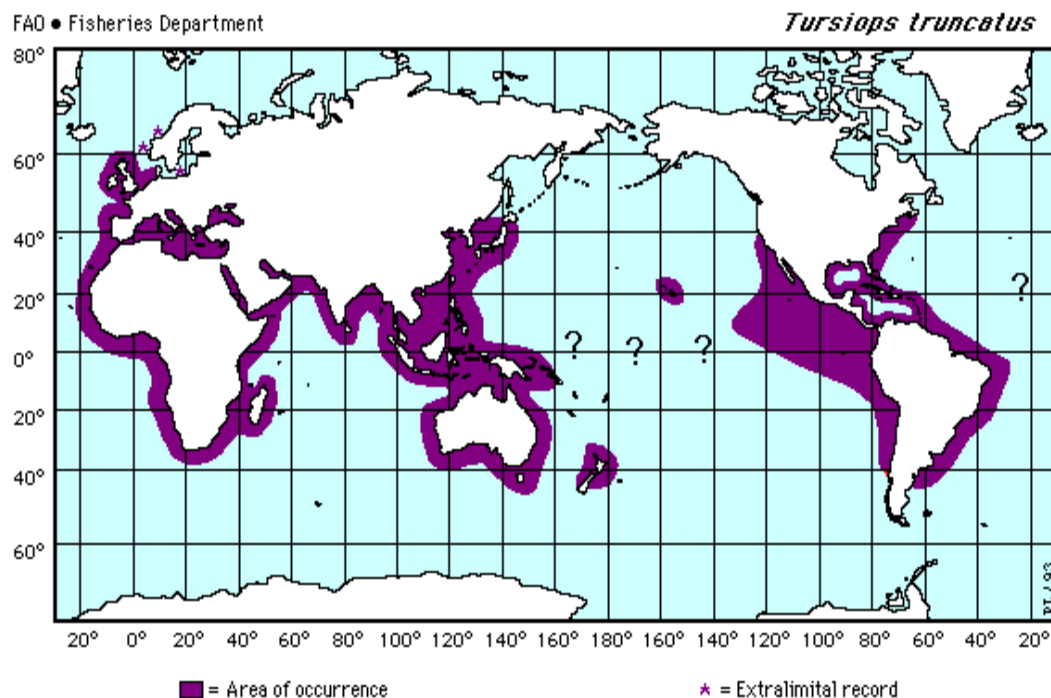


Figure 1. *Tursiops truncatus* worldwide distribution according to Jefferson et al. 1996.

1.4. The studied population

The Scottish northeast population of bottlenose dolphins, often referred to as the Moray Firth population is small and lies at the extreme of the distribution of the species (Wilson 1995). Its distribution has been documented from the Moray Firth in the north to Fife Ness in the south (Wilson et al. 2004). Wilson (1995) gathered historical records of naturalists of the 1800s and it appears

that bottlenose dolphins were not common in the Moray Firth until the very end of the 17th century.

Up in the Moray Firth the presence of bottlenose dolphins has been documented all year round with high density peaks in the summer. By traditional photo-identification techniques, 115 individuals have been identified as residents. The group size can fluctuate from 2-46 with an average of 6.45 and it is correlated to the amount and distribution of the prey (Wilson 1995). Outside the Moray Firth surveys around Aberdeen harbour have documented the presence of bottlenose dolphins mostly displaying foraging behaviour (Sini et al. 2005). Its presence around Fife Ness seems to be restricted to the summer period and at least 65 individuals have been identified, although the population could be composed of up to 130 different individuals (Quick 2006).

Bottlenose dolphins show different association patterns. They can form long lasting behavioural associations, or short acquaintances that can last a few days (Gero et al. 2005). The individuals in the Moray Firth do not show any strong, long lasting association, males seem to associate with different individuals of both sexes more often than females do and tend to form bigger groups (Wilson 1995). On a bigger scale this population appears to be stratified in two groups that use the same habitat at different times, suggesting some kind of competition between social groups or communities (Lusseau et al. 2006; Wilson et al. 1997a) connected via a limited number of individuals (Lusseau et al. 2006).

Population structure studies of dolphins inhabiting UK waters suggest that the Moray Firth population is isolated from its neighbouring populations (Nichols et al. 2007; Parsons et al. 2002), but is genetically closer to the population of Wales than to its closer neighbours at the West coast of Scotland (Parsons et al. 2002). The mitochondrial genetic diversity values of the Moray

Firth population were much lower than the ones of the other UK populations and other populations around the world (Parsons et al. 2002).

This decrease in genetic diversity and the isolation and small size of the East Coast Scottish population, raises concerns about the possibility of inbreeding depression that could have detrimental effects. Wilson et al. (1997b) found that 95% of the dolphins sampled in four years showed some kind of skin lesion and 6% showed deformities; these lesions were more extensive in female adults and calves than in male adults. When studying several populations with skin lesions worldwide, there was no correlation between these lesions and contaminant levels the populations is exposed to, but there was a correlation with low temperature and low salinity (Wilson et al. 1999). This suggests that the habitat these animals occupy can cause physiological stress that makes the population vulnerable (Wilson et al. 1999).

Populations around the UK occupying the extreme range of the distribution of the species seem to be under physiological stress; they have a small population size and seem to show local adaptations. To what extent are these facts a cause of concern? Nichols et al. (2007) investigated the genetic origins and population structure of a group of bottlenose dolphin bones found in the Northeast of England (Flixborough). These individuals showed the dominant mitochondrial haplotype of the Eastern Scottish population, but they were differentiated as a population by microsatellites (Nichols et al. 2007). Nichols et al. (2007) suggested that local habitat dependence is related to regional genetic structure in these populations. The fact that the Flixborough population went extinct more than 100 years ago and has not been replaced, could be seen as evidence that bottlenose dolphin populations living in these waters might constitute a declining meta-population (Nichols et al. 2007).

1.5. Methodological considerations

1.5.1 Phylogeography

Phylogeography is a field that studies the geographic distribution of the genealogical lineages of different species (Avice 2000). It studies the time and space of several genes of interest that may be used to know the actual distribution and genetic structure observed in natural populations. The analysis and interpretation of lineage distributions requires the integration of several fields like population genetics, molecular genetics, ethology, demography, phylogenetic biology, paleontology and historical geography (Avice 2000).

Population genetics has grown widely in the last 15 years due to the introduction of new DNA based technologies. Sequence analysis of mitochondrial DNA (mtDNA) and the identification of nuclear microsatellite genotypes have become two standard tools in most of the animal genetic research, since they allow us to make inferences of phylogenetic relationships, gene flow, phylogeographic patterns and genetic variability (microsatellites and mtDNA), as well as fine analyses of population structure (microsatellites) (Sundqvist et al. 2001).

1.5.2. Mitochondrial DNA (mtDNA)

Mitochondrial DNA has been widely used in phylogeographic studies and it is considered one of the best markers due to its high mutation rate, lack of recombination and maternal inheritance (Avice 2000). Different sites in different mitochondrial genes evolve at different rates within several species lineages. One of the most used regions of the mitochondrial genome, for looking at differences between populations of the same species, is the control region containing the D-loop. This region shows a rapid evolution and exhibits high levels of intraspecific polymorphism. Some authors suggest that

its substitution rate may be three to five times higher than the rest of the mitochondrial genome (Avice 2000). The substitution rate for cetaceans compared to humans seems to be one degree of magnitude lower, but similar to interspecific rates shown in primates and rodents (Hoelzel et al. 1991) (Hoelzel et al. 1991). Insertions and deletions are not as common in cetacean control regions as they are in other taxa. Point mutations seem to play the most important role in cetacean control region evolution (Hoelzel et al. 1991).

In spite of this high polymorphism the central position of the control region shows a similar nucleotide composition between different species and it does not diverge faster than the rest of the protein-coding genes of the mitochondrial genome (Hoelzel et al. 1991). This feature makes inter and some intraspecific comparisons of the control region plausible and quite informative.

1.5.3. Nuclear genetic markers: Microsatellites.

Microsatellites also known as STR, SSR and SLP (Short Tandem Repeats, Simple Sequence Repeats and Single Strand Length Polymorphisms) (Bruford & Wayne 1993; Tautz & Renz. 1984) are small DNA fragments widely spread in the eukaryotic genomes (Tautz & Renz 1984). These fragments consist of motifs of one to six nucleotides that repeat themselves in tandem up to 60 times or more (Goldstein & Pollock 1997). In eukaryotes these fragments can be found every 10 Kb in the DNA sequence and they constitute approximately 5% of the genome (Tautz 1989).

One of the advantages of these markers is that the alleles are scored by their sizes due to their molecular weights (Palsbøll et al. 1997). The length of these fragments ranges between 50 to 300 bp, for this reason it is quite easy to

observe them in common polyacrilamide gels and detect small differences between them (Tautz 1989).

Microsatellites are extremely variable in the number of alleles reported due to the mutations in the number of repeated units by insertion or deletion (Tautz 1993 cited in: Nauta and Wissing 1996). The mutation rate of microsatellite *loci* is very high and seems to range between 10^{-5} and 10^{-2} (Weber & Wong 1993). This characteristic and the fact that they are relatively easy to screen have made them quite popular in population genetics, relatedness, parentage and individual identification studies (Goldstein & Pollock 1997).

These markers have become quite commonly used in cetacean research. Several studies have characterized nuclear microsatellites for their use in population studies (Valsecchi and Amos, 1996; Shinohara et al. 1997; Rooney et al. 1999; Hoelzel et al. 1998b; Krutzen et al. 2001). This makes it easier to find polymorphic *loci* in specific populations and gives us the opportunity to compare patterns in different locations from different studies. Most of the microsatellites in cetaceans have been developed to amplify dinucleotide motifs. Dinucleotide microsatellites scoring have been found to convey several mistakes while genotyping that result in large amount of errors in assigning paternity in wild populations (Hoffman & Amos 2005) mainly due to the presence of stuttering bands that are a common by-product of PCR amplification (Litt et al. 1993). For these reasons tetranucleotide markers are now becoming more widely used in the recent years and a couple of studies have developed them for cetaceans (Coughlan et al. 2006; Nater et al. 2009). Nater et al. (2009) developed a set of 19 tetranucleotide markers for bottlenose dolphins and compared their accuracy to previous dinucleotide microsatellites. They found a four-fold increase in scoring accuracy on the tetranucleotides but a decrease in the polymorphism of the markers.

Aims of my PhD study:

The main aim of my PhD study was to investigate how the social patterns of bottlenose dolphins in the East Scottish population of bottlenose dolphins would be affecting the genetic patterns observed in the same. To achieve this objective I obtained biopsy samples and photo-identification data from the East Scottish population of bottlenose dolphins during the summer periods of 2006 and 2007.

I employed molecular techniques to confirm the sex of each sample and to investigate relatedness between the biopsied individuals. The association patterns of the East Scottish population were described including data from previous studies and a correlation between association and relatedness was investigated. The presence of strong bonds between female relatives in cohesive groups along with the presence of adult male alliances was expected. Male alliances are a common reproductive strategy that has been documented in other populations of bottlenose dolphins around the world. Contrary to our expectations no correlations were found between association and relatedness (Chapter 3) and male alliances are not present in the population (Chapter 2).

Finally I analyzed the genetic structure of bottlenose dolphin populations around UK waters with mitochondrial DNA and nuclear microsatellites. Previous studies found an alarming decrease in the mitochondrial genetic diversity of the East Scottish population. They also found a strong isolation of the East Scottish population from the neighbouring populations in the West Coast of Scotland. The sample size of these studies was considerably small and all the samples came from strandings. I expected that patterns of gene flow and an increase in genetic diversity would be revealed with a more thorough sampling.

Chapter 2 Population Structure of bottlenose dolphins around UK waters.

2.1 Introduction:

Natural populations are generally structured in subpopulations, interconnected by different levels of migration (Perrin & Mazalov 2000). Gene flow is the main force that determines subpopulation structure and how independently they evolve from each other (Slatkin 1987). An understanding of this structure is essential to create effective population management and conservation policies (O’Corry-Crowe et al. 1997), since subpopulations can be separated by varying degrees of genetic isolation. Traditional population genetic studies have employed genetic markers to uncover the dispersal dynamics of the population and how this is reflected in the population structure.

The study of genetic subdivision patterns among cetaceans is difficult because cetaceans are capable of traveling long distances (Escorza-Treviño & Dizon 2000) and have large habitat ranges with no evident barriers to gene flow besides water temperature, marine topography, (Würsig & Würsig 1979) productivity and surface features such as salinity (Natoli et al. 2005).

Bottlenose dolphins, *Tursiops truncatus* (Montagu 1821) along with other odontocete species show a promiscuous breeding system (Wells & Scott 1999). In the promiscuous or polygynous breeding systems the male’s reproductive success is limited by the availability of females, while the fitness of the females is limited by its capacity to process resources. This results in a small male contribution to parental care and pronounced competition for females as well as male dispersal (Perrin & Mazalov 2000).

Patterns of dispersal are well differentiated between the sexes in a variety of organisms (Greenwood 1980). Although male biased dispersal is common in mammals and has been studied for several cetacean species with molecular markers (Escorza-Treviño & Dizon 2000; Lyrholm et al. 1999; Moller & Beheregaray 2004; O'Corry-Crowe et al. 1997), recent studies of bottlenose dolphins have found that both sexes can be philopatric to some extent, showing fine scale structure related to water temperature, salinity and productivity (Natoli et al. 2005).

Among cetaceans intraspecific differentiation may be sympatric or parapatric (Hoelzel 1998). It seems that the main forces driving cetacean population differentiation are the specializations that result from their foraging behaviour (Hoelzel 1998). The evolution of these traits is influenced by three main ecological aspects: place of birth, diet and foraging locations (Connor et al. 2000a) .

In bottlenose dolphin populations, two different ecotypes have been documented. In the Western North Atlantic “coastal” bottlenose dolphins have smaller sizes than the “pelagic” ones. Significant differences in measurements that are related to the size, mainly total length and skull length, were found between the two ecotypes, but with an extensive overlap in the measurements from both ecotypes (Mead & Potter 1995).

This pattern is reversed in the bottlenose dolphin populations of the Eastern North Pacific, where the morphological differences are so evident that coastal and pelagic dolphins have been considered to be different species. The “pelagic” form (*T. nuuanu*) is significantly smaller in several cranial measurements and it feeds on epipelagic fish and cephalopods, while the coastal form (*T. gilli*) is bigger and it feeds on coastal fish species from the Sciaenidea and Embiotocidae family (Walker 1981).

Hoelzel et al. (1998) used mitochondrial and nuclear genetic markers to find out to what extent these “coastal” and “pelagic” populations were genetically divergent in the North Atlantic. They found strong significant differences between the two ecotypes with both markers and a reduced genetic diversity among the “coastal” populations compared to the “pelagic” ones.

Pronounced genetic differences are not exclusive to foraging specializations in odontocetes. Dowling and Brown (1993) analysed RFLP's (Restriction Fragment Length Polymorphisms) for the mitochondrial DNA (mtDNA) control region of *Tursiops truncatus*, of neighbouring “coastal” populations and found significant differences between the stocks of the Atlantic Ocean and the Gulf of Mexico divided by the Florida Peninsula, but not between putative populations from the northeast of Florida or between populations from the southwest of Massachussets. More recently the population structure of resident “coastal” stocks from Sarasota Bay, Tampa Bay, Charlotte Bay and Matagorda Bay was analyzed using the control region of the mtDNA and nine microsatellite loci. Here, Sellas et al. (2005) found a strong population subdivision with both markers for both sexes, indicating a strong phylopatriy of males and females and a restricted gene flow between close, coastal, neighbouring populations.

A similarly restricted flow for both sexes was found when assessing the population structure of bottlenose dolphins worldwide with nine nuclear microsatellites and mtDNA control region sequences for individuals from the northern Gulf of Mexico, Western North Atlantic, Eastern North Pacific, Mediterranean Sea, West Atlantic, Bahamas, South Africa and China. All *Tursiops truncatus* populations showed a great population differentiation and the genetic division between coastal and pelagic populations were also confirmed, as it was expected. Between the groups of *Tursiops aduncus* they found a similar high level of divergence, therefore suggesting a third species of *Tursiops aduncus* in South Africa (Natoli et al. 2004). The suggested

philopatry of both sexes was also displayed by the populations of bottlenose dolphins from the Black Sea to the eastern North Atlantic, showing a correspondence between the population structure and the use of habitat (Natoli et al. 2005).

Patterns of dispersal are well differentiated between sexes in a variety of organisms (Greenwood 1980). The resulting patterns of gene flow are of great importance to elucidate the phylogeographic pattern of the species (Avice 2000). Although the latter studies in bottlenose dolphins show philopatric patterns present in both sexes, male sex-biased dispersal has been documented for several cetacean species by means of molecular analysis. This includes belugas, sperm whales and Dall's porpoises (O'Corry-Crowe et al. 1997; Lyrholm et al. 1999; Escorza-Trevino and Dizon 2000) and bottlenose dolphin (*Tursiops aduncus*) populations of southeastern Australia (Möller & Beheregaray 2004).

A previous genetic study of the bottlenose dolphin populations of the United Kingdom, analysed mtDNA sequences from 29 stranded animals. This study revealed that the Moray Firth population was genetically closer to the population of Wales than to the neighbouring population of the west coast of Scotland. The genetic diversity values of the Moray Firth population were much lower than the ones of other UK populations and other populations in the UK and worldwide (Parsons et al. 2002).

This reduced amount of genetic diversity both in 171bp of the mitochondrial DNA control region and five microsatellite markers in animals of the East Coast of Scotland was also found by Nichols et al. (2007) while looking at the population structure of the UK extant populations of bottlenose dolphins in relation to an extinct population found in Flixborough. Nichols et al. (2007) also found a pronounced genetic isolation of the East Coast of Scotland from

the neighbouring populations with both mtDNA and microsatellites. They also found that the Flixborough population was mostly related to the East Coast population and other populations around the UK, but also much differentiated from them. They suggested that local adaptations in these populations that are located at the northern extreme of the distribution of the species are very strong and that the gene flow is much reduced.

In this study the largest set of cumulative samples to date from the East Coast of Scotland and neighbouring populations was gathered. This collection included both stranded samples and biopsies from wild animals.

The aim of this study is to fine tune the relationships of the bottlenose dolphin populations around the UK. Previous studies have used only stranded samples which origins could be inaccurate. They rather suffered of lack of sample size or they pooled together samples from different populations in order to achieve significance. In this study I try to establish if the East Coast of Scotland population is isolated from the neighbouring populations and to ascertain the implications that this may have on its conservation.

2.2 Methods:

2.2.1. Sample origins and DNA extractions

One-hundred eleven samples from strandings and biopsies were collected from four putative populations in the United Kingdom waters: East Coast of Scotland, West Coast of Scotland, Wales and English Channel (Fig. 1).

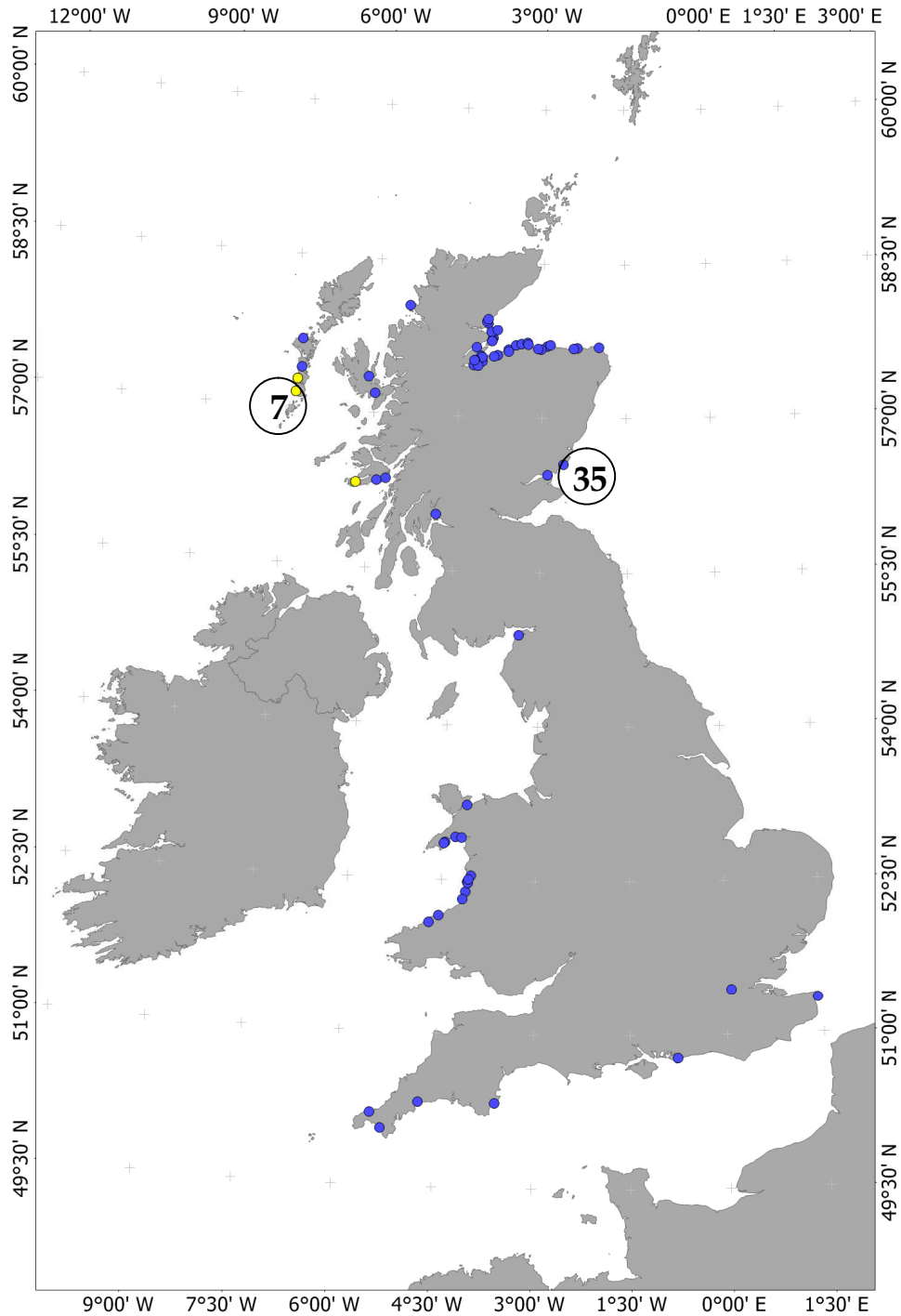


Figure 1. A map of Great Britain and Ireland showing the location of samples from strandings included in this study. Above the general area where the biopsies took place, the total number of biopsies is shown in circles. West Coast strandings in yellow circles are from individuals that shared the same haplotype with the Barra biopsies. The total number of samples from the four putative populations is: a) East Scotland 69 individuals, b) West Scotland 19 individuals, c) Wales 15 individuals and d) English Channel 7 individuals.

Sixty-nine stranding samples came from tissue donated by the Scottish Strandings Coordinator in Inverness and the Marine Mammal Strandings Research Coordinator in London. Thirty-five biopsy samples from the East Coast of Scotland were collected as described in Chapter 3 and seven West Coast biopsy samples were collected only for purposes of genetic structure studies. The sex of the samples was given by the Stranding Network or determined with molecular techniques (Table 1) as described in Chapter 3.

Table 1. Details of one-hundred eleven samples collected in this study. Number and gender of the samples analyzed for the four populations.

Population	Strandings	Biopsies	Females	Males	Unknown
East Coast	35	35	24	41	5
West Coast	12	7	10	5	4
Wales	15		8	5	2
English Channel	7		4	3	
Total	69	42	46	54	11

All samples were kept in ethanol 70% at -70°C. Due to the heterogeneity of the tissue samples, DNA was extracted by three different techniques. The standard phenol-chlorophorm technique (Sambrook et al. 1989) was used for most of the cases as a first approach. The standard salt-saturated extraction technique (Sunnucks & Hales 1996) was used when the first technique was unsuccessful. If the tissue sample was very small a Forensic kit for Genomic DNA Isolation (Invisorb) was used. One-hundred and ten DNA samples had sufficient quality to be analyzed.

2.2.2. Mitochondrial DNA

A 660 bp section of the control region was amplified for 110 samples using the primers: Rev (5'GTGACGGGGCCTTTCTAA 3') (LeDuc et al. 1999) and F2 (5'CTC ACC ACC AAC ACC CAA AG 3'). The F2 primer was designed with Primer 3 (<http://primer3.sourceforge.net/>) from a *Tursiops truncatus* sequence (AY963625) to obtain a longer fragment from the one already published by Parsons et al., (2002). Polymerase chain reaction conditions were as follow: 150 μ M dNTPs, 1.5 mM MgCl₂, 20 mM Tris-HCl pH 8.0, 50 mM KCl, 0.3 μ M of each primer, 1.25 U/ μ L of Taq (Bioline) and 20 ng of DNA for a 25 μ L total reaction. PCR cycling profile: 4min at 95 °C, 30 cycles of 45 secs at 94°C, 1 min at 55.8°C and 1 min at 72 °C, followed by a final extension of 5 min at 72°C.

PCR products were purified with a QIAGEN QIAquick gel extraction kit and quantified for automated sequencing. Individuals were sequenced in both directions (*forward and reverse*) to verify the identity of each nucleotide in several cases where the sequences were not of high quality. Sequences were edited, checked and aligned by eye with BIOEDIT 7.0.5.3.

2.2.2.1 Genetic diversity

Nucleotide (π) and haplotypic (h) diversities (Nei 1987) were calculated for each population with the program ARLEQUIN 2.0 (Schneider et al. 2000). The population differentiation was measured with an analysis of molecular variance AMOVA (Excoffier et al. 1992) performed by Arlequin ver 3.1, along with the pairwise comparison of population differentiation indices F_{ST} (Wright 1965) and ϕ_{ST} between all the populations analyzed. The Tamura-Nei genetic distance model (Tamura & Nei, 1993) was used to obtain ϕ_{ST} estimates.

2.2.2.2. Phylogeographical patterns

To organize the haplotypes observed in our populations in a way that portrays the evolutionary steps between them, a haplotypic network was created with the program TCS 1.18 (Clement et al. 2000). The assumption of this approach is that if an unknown mutation causing a phenotypic effect occurred at some point in the evolutionary history of the population, it would be embedded within the same historical structure represented by the cladogram (Templeton et al. 1992). TCS calculates the frequencies of the haplotypes and creates a matrix of pairwise comparisons among them for which the probability of parsimony is calculated (Clement et al. 2000). The algorithm developed by Templeton et al. (1992) estimates all the possible cladograms with a high probability (≥ 0.95) of being true. The probabilities are higher when the number of changes between haplotypes is smaller and the probability decreases as the differences between haplotypes increase (Templeton et al. 1992). This method is suitable for intra-specific studies and it has been used to infer population genealogies particularly when they show low levels of divergence (Clement et al. 2000).

The different haplotypes across all the populations were compiled using the program COLLAPSE 1.2 (Posada © 1998-2006). These haplotypes were aligned with *Tursiops truncatus* haplotypes obtained from GenBank representing the following regions: Portugal (Tt-PO), Mediterranean (Med), Baltic Sea (BSea) and ENA (Eastern North Atlantic). Sequences from other species, were used as outgroups in the alignment, to resolve the relationships in a better way: *Sousa chinensis* (Schinensis), *Stenella*, *Delphinus capensis* (Dcapensis), *Grampus griseus* (Ggriseus), 2 haplotypes of *Orcinus orca* (Oorca) and 2 haplotypes of *Tursiops aduncus* (Taduncus). All accession numbers of sequences obtained from the GenBank are in Appendix D.

It has been suggested that when the evolutionary period represented by a cladogram is short, like it is in the case of intra-specific processes, maximum likelihood and maximum parsimony tend to give very similar results (Sober 1983 in Templeton et al. 1992). For this reason we constructed one tree with parsimony methods and another one with Bayesian ones. A parsimony consensus tree was constructed with PAUP (4.0 beta10) using 1000 bootstrap replicates and *Orcinus orca* as the outgroup.

The individual haplotypes were analyzed to obtain a substitution model for the amplified region with the programs MODELTEST 3.05 (Posada & Crandall 1998) and Modelgenerator v0.85 (Keane et al. 2006). The substitution model that best fit the data according to Modeltest hierarchical likelihood ratio test and Modelgenerator Bayesian information criterion (BIC) was Trn+I+G (Tamura & Nei 1993). This model takes into account different rates of substitution between nucleotides: [A-C],[A-G],[A-T],[C-G],[C-T] and [G-T] (rate matrix) and different nucleotide frequencies. The rates among the sites are modeled using the gamma distribution. Thus a gamma parameter is required along with a proportion of invariable sites (I).

The probability of observing the data conditional to the phylogenetic model is the likelihood function, which is calculated assuming a model of character changes (Huelsenbeck & Ronquist 2001). The parameter for the likelihood model 'lset' was set as Nst=6, this model allows all the substitution rates to be different as is the case in the Trn+I+G model found in Modeltest. The model outcome had a proportion of invariable sites (I)= 0.6100, a gamma parameter of (G) = 0.5479 , a rate matrix= 1.0000 17.9388 1.0000 1.0000 40.1796.

All the parameters obtained from Modeltest were fed into Mr Bayes 3.1 (Huelsenbeck & Ronquist 2001) to construct a Bayesian consensus tree. Several runs were performed with different sampling frequencies, to determine if the sampling frequency showed autocorrelation between

samples. An autocorrelation test of the Ln function from the parameters obtained was carried out with the Statistical Program R (2005). The sampling of each tree was done every 20 000 generations. The initial 2 000 trees converged and were discarded (burnin), 2000000 generations were simulated with just one hot chain. The two *O. orca* haplotypes were designated as outgroups.

2.2.3. Microsatellites

Twenty previously reported polymorphic nuclear microsatellite loci were analyzed for all 110 samples. The original source of the microsatellites and the PCR details are shown in Table 1 (Appendix). The twenty microsatellites were amplified with a fluorescent dye and automatically sequenced (Beckman Coulter). The markers were amplified in 3 loci groups with a Multiplex PCR kit from (QIAGEN) with conditions shown in Table 2.

Table 2. Multiplex PCR Loci Groups Characteristics. Each Locus Group (LG) shows Locus name, type of dye and concentration of dye are shown.

LG1

LG2

Locus	DYE	[DYE]	Locus	DYE	[DYE]
TexVet5	D4	0.12 μ M	Tur4_80	D4	0.16 μ M
TexVet7	D3	0.8 μ M	MK9	D2	0.8 μ M
D08	D3	0.6 μ M	EV1	D3	0.8 μ M
D22	D4	0.12 μ M	Tur_91	D4	0.16 μ M
MK6	D2	0.8 μ M	Tur_117	D4	0.16 μ M
			MK8	D4	0.08 μ M

LG3

Locus	DYE	[DYE]
Tur105	D3	0.8 μ M
Dde72	D4	0.16 μ M
Tur138	D3	0.8 μ M
Dde84	D4	0.16 μ M
Dde70	D3	0.8 μ M
Dde61	D2	0.8 μ M

PCR reactions consisted of 10-20 ng of genomic DNA, 5 µl of Multiplex Mix and 3 µl of primer mix in a 10 µl reaction. The PCR profile was as follows: 95°C for 15 min followed by 30 cycles of 94°C for 30sec, 60°C for 90 sec and 71°C for 45sec, with a final extension of 72°C for 2 min.

Genotyping error was calculated separately for biopsies and strandings by randomly re-amplifying between 10% and 50% of the individuals for each locus. Each individual repeat was genotyped at least once and up to 6 times. If both allele lengths were identical each time, it was counted as two matches, but if either allele was different, it was considered two mismatches. The number of mismatches was divided by the total number of comparisons to obtain the error percentage for each locus in both biopsies and strandings. Finally all loci were run in Micro-checker (Van Oosterhout et al. 2004) to check them for null alleles, misgenotyping and stutter bands.

2.2.3.1. Genetic diversity

The genetic diversity was calculated as expected and observed heterozygosity (H_E and H_O) with the program (Genetix v 4.03). Deviation from HW equilibrium and the probability test were calculated with GENEPOP v. 3.1d (Raymond & Rousset 1995b). The allelic richness was calculated with FSTAT 2.9.3.2 (Goudet 1995).

2.2.3.2. Population Structure

Pairwise comparisons of genetic differentiation (F_{ST}) were conducted with the program GENEPOP and FSTAT was used to test the significance of the resulting estimates. Pairwise comparisons of genetic differentiation for Rho_{ST} were calculated with RstCalc (Goodman 1997). Jost (2008) pointed out that

F_{ST} is based to show high levels of differentiation when loci show high values of genetic diversity (high values of heterozygosity) and he developed a new measure to cope with that problem (D_{EST}). D_{EST} was calculated with the program SMOGD (Crawford 2009) and compared with both F_{ST} and Rho_{ST} . The linkage disequilibrium for each locus was calculated with GENEPOP. A sequential Bonferroni correction (Rice 1989c) was applied later to assess significance values.

The patterns of genetic structure were analyzed with Structure 2.3.1 (Pritchard et al. 2000). This program uses a Bayesian clustering analysis to determine the number of populations (K) observed according to the data and it determines the posterior probability of each single individual belonging to a particular population. The burn in period was set to 50 000 iterations and the probability estimates were determined using 1 000 000 Markov chain Monte Carlo (MCMC) iterations. Runs were conducted with K set from 1 to 10 with 10 runs for each value of K. Two separate tests were conducted with two different models: the no admixture model and the admixture model. The no-admixture model assumes that all the individuals come from the same population K; this model is good at detecting subtle population structure. The admixture model assumes that the individuals from all the populations could have a common ancestor and it is good at dealing with hybrid zones. When running the admixture model we assigned individuals to five putative populations: Moray Firth, Outer Community, West Coast, Wales and English Channel, to confirm if the sampling area is informative. We divided the East Coast of Scotland in Moray Firth and Outer Community, to test if the separation found by Lusseau et al. (2006), with a network analysis was consistent with the genetic pattern. The samples representing the Moray Firth were all from strandings and the most of the Outer Moray Firth samples were the biopsies obtained in this study (St Andrews Bay and stranding samples from outside the Moray Firth). The West Coast of Scotland samples also comprised both biopsies from the population of Barra and strandings from

other locations. We did not divide the West Coast of Scotland samples due to the small number of biopsies from the region and the overall small sample size. Finally Structure was run with the admixture model, correlated frequencies, burnin of 11000, 1000000 repetitions and 5 iterations for each value of K from K=1 to K=8. This run included only the East Scottish samples to detect any structure within the population.

2.2.3.2.1. Estimation of parameter K

The power of the Bayesian algorithm to obtain the true K from the log probability of the data $\text{LnP}(D)$, has not been well documented in a scenario with a non-homogeneous dispersal patterns. Evanno et al. (2005) developed a method to calculate an *ad hoc* statistic called ΔK to correct this problem by obtaining the second order rate of change of $\text{LnP}(D)$ between the values of K. This statistic (ΔK) can be obtained following 4 steps.

- a) The means and standard deviation (SD) of the log probability for each K 1 to 8 were obtained $L'(K)$.
- b) The first order rate of change was calculated as $L''(K) = L(K) - L(K-1)$
- c) Absolute values of the second order rate of change were calculated as $|L''(K)| = |L'(K+1) - L'(K)|$
- d) ΔK was calculated as the absolute values of the second order rate of change divided by the standard deviation of each K following the following formula $\Delta K = L''K / \text{SD } L(K)$. The modal value of this distribution is the true K.

2.2.3.3. Estimation of migration rates and sex biased dispersal

To assess the levels of present migration, a Bayesian multilocus approach employed by BayesAss 1.3 (Wilson & Rannala 2003) was used. Three million

iterations, a sampling frequency of 2000 and burn-in of 999999 were the parameters for the analysis. The stabilization of the log likelihood values within the period set by the burnin was checked and the mean and variance of the posterior probabilities for the migration rates were obtained. Sex-biased dispersal was calculated with FSTAT by calculating pairwise F_{ST} comparisons for females and males separately between all populations using 10,000 randomizations with a one-tailed test.

2.2.3.4. Relatedness between populations

As a final strategy to elucidate the relationship between the populations analyzed we used the Relatedness analyses explained in detail in Chapter 3. Pairwise symmetric relatedness was calculated for all the 101 individuals from the four populations analyzed with the program RE-RAT (Schwacke et al. 2005). Re-RAT calculated R using the Queller and Goodnight (1989) index with a jackknife over loci of 100 simulations. Average relatedness for each population and for classes of males and females were calculated in the same way.

A distance matrix was obtained by subtracting 1 from each value of R for the pairwise comparison between individuals. With this distance matrix a UPGMA (Unweighted Pair Group Method with Arithmetic mean) tree was constructed with the program Neighbor that is a part of the software PHYLIP (Felsenstein 2005).

2.3. Results:

2.3.1. Mitochondrial DNA

2.3.1.1. Genetic diversity

A 507 bp section of the control region of 87 samples from 4 populations was sequenced. The DNA in the remaining samples was too degraded to be sequenced. Twelve different haplotypes were found (Table 2). Between haplotypes 1, 2, 3, 5 and 7 there are just one or two differences, while the remaining haplotypes had multiple substitutions from haplotype 1.

Table 2. The twelve haplotypes. The position in the sequence where the substitutions occurred is shown in the top of the table, when the nucleotides remain the same it is indicated by a "-".

Position	9	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	4	4
	0	0	8	9	3	5	6	7	7	7	8	4	6	8	8	8	4	7
		8	6	7	7	7	9	0	1	4	6	9	2	3	4	5	5	0
Hap1	C	C	T	T	C	A	T	T	C	C	C	T	C	C	A	T	T	C
Hap2	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
Hap3	-	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	-
Hap4	T	-	C	C	T	-	-	C	T	-	-	-	T	-	C	-	C	-
Hap5	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap6	T	-	-	C	T	-	-	C	T	-	T	-	T	-	C	-	-	T
Hap7	-	-	-	-	-	G	-	-	-	-	-	-	-	T	-	-	-	-
Hap8	T	-	-	C	T	-	C	C	T	-	T	-	T	-	C	-	-	T
Hap9	T	-	-	C	-	-	-	C	-	-	-	-	T	T	C	-	-	T
Hap10	T	-	-	C	T	-	-	C	T	-	T	C	T	-	C	C	-	T
Hap11	T	-	-	C	-	-	-	C	T	-	-	-	T	-	C	-	C	-
Hap12	T	-	-	C	T	-	-	C	T	-	T	-	T	-	C	-	-	-

Fifty-six individuals were analysed from the East Coast population and just 3 haplotypes were found (Table 3). Most of the individuals had Hap 1 (n=44), followed by Hap 2 (n=11). Hap 3 was only found in one individual. Three different haplotypes were found in the nine individuals for Wales. Six of these had the most common haplotype of the East Coast of Scotland (Hap1) and also the two more different haplotypes Hap4 (exclusive for the

population) and Hap7. Animals from the English Channel had five different haplotypes in the six samples analyzed. Two of them were from Hap1 and one from Hap2, one haplotypes was shared with Wales (Hap 7) and had two exclusive ones (Hap5 and Hap6). Animals from the West Coast of Scotland had a total of seven haplotypes. Just one individual showed Hap1 and one Hap2. The most common, and exclusive haplotype, was Hap 8 with 10 individuals. This haplotype was unique to the population of the West Coast of Scotland. Hap9, Hap10, Hap11 and Hap12 were also found exclusively at the West Coast of Scotland.

Table 3. Number and distribution of the Mitochondrial DNA haplotypes.

Haplotype	East Coast	Wales	English Channel	West Coast	Total Ind per Hap
Hap1 (B-01)	44	6	2	1	53
Hap2 (B-02)	11		1	1	13
Hap3 (B-21)	1				1
Hap4 (SW-1)		1			1
Hap5 (SW2007/84)			1		1
Hap6 (SW2007/201)			1		1
Hap7 (SW2006/98)		2	1		3
Hap8 (M160/00)				10	10
Hap9 (M167/98)				1	1
Hap10 (M1924/98)				1	1
Hap11 (M146/01)				1	1
Hap12 (M32/08)				1	1
Total Individuals per population	56	9	6	16	TOTAL= 87

Despite the considerably larger sample size for the East Coast of Scotland, the population had the lowest gene and nucleotide diversity among the four populations. The English Channel had the smallest sample size and the highest genetic diversity scores, followed by West Scotland and Wales (Table 4).

Table 4. Mitochondrial DNA diversity. Number of samples, haplotypes, polymorphic sites, gene and nucleotide diversity are shown. Gene or Haplotype diversity ($h \pm$ S.D) as well as Nucleotide diversity π (\pm S.D) within each population.

Population	East Scotland	English Channel	Wales	West Scotland
No of samples	56	6	9	16
No of haplotypes	3	5	3	7
Polymorphic sites	2	12	11	14
Gene diversity (h)	0.3500 \pm 0.0670	0.9333 \pm 0.1217	0.5556 \pm 0.1653	0.6250 \pm 0.1390
Nucleotide diversity (π)	0.000747 \pm 0.000798	0.008284 \pm 0.005523	0.005479 \pm 0.003622	0.007495 \pm 0.004460

2.3.1.2. Population Structure and Phylogeography

Pairwise comparisons of the population differentiation indices (F_{st} and ϕ_{st}) were obtained with Arlequin ver 3.1 (Table 5). The genetic distance model used to obtain ϕ_{st} was Tamura-Nei (Tamura and Nei 1993). The strongest population differences with both indices (F_{st} and ϕ_{st}) were between East and West Scotland, followed by West Scotland *vs* Wales, West Scotland *vs* English Channel and English Channel *vs* East Scotland. Between East Scotland *vs* Wales just the $\phi_{st}=0.85958$ was significant (Table 5).

Table 5. Pairwise population differentiation for the section of mitochondrial DNA sequence. F_{ST} values below the diagonal and ϕ_{ST} above the diagonal. (* $P < 0.05$, ** $P < 0.001$, * $P < 0.0001$). Number of permutation for P values =110.**

Population	East Scot n=56	Wales n=9	English Channel n=6	West Scot n=16
East Scotland	-	* 0.15003	** 0.27106	*** 0.85958
Wales	0.08100	-	-0.10754	*** 0.60348
English Channel	* 0.21290	0.01072	-	*** 0.51472
West Scotland	*** 0.52794	*** 0.37874	** 0.22723	-

A better representation of the relationships between the haplotypes is shown in the haplotype network in Fig. 2. The network shows a very strong divergence between the East Coast (blue) and the West Coast of Scotland (red) haplotypes. Haplotype B-01 (Hap1) is present in all the populations and is the most common (Table 3). Haplotype B-02 (Hap2) seems to be the most ancient haplotype (square), showing more connections to other haplotypes than any other haplotype and it is also present in a large number of individuals. All the haplotypes present in the East Coast (blue) are connected by just one substitution, showing a lack of genetic diversity in this population. The West Coast (red) shows a larger number of haplotypes separated by missing haplotypes (empty dots). The Welsh and English single haplotypes are spread at both sides of the network.

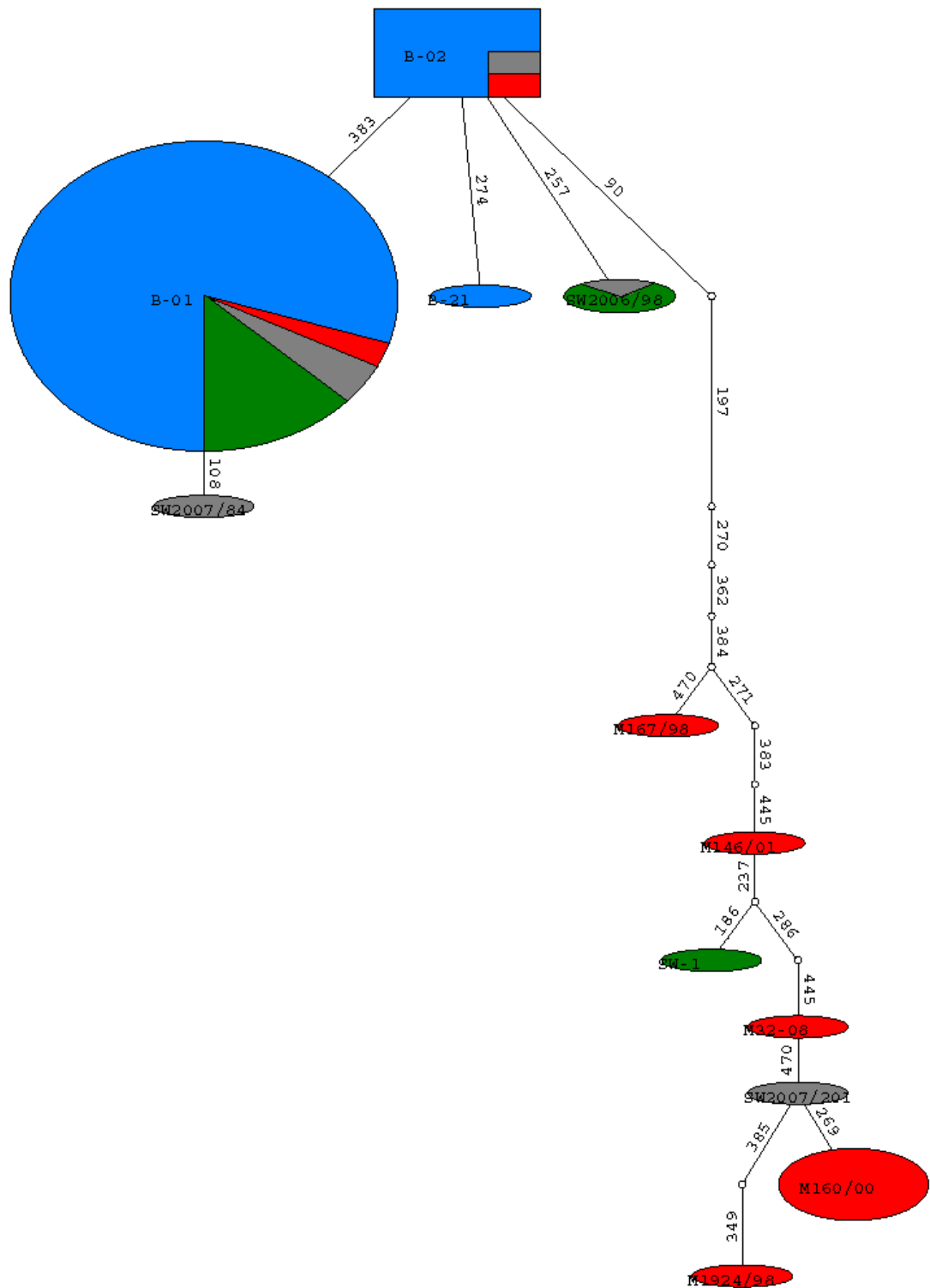


Figure 2. Haplotype network showing relationships between the haplotypes, populations are represented by colours a) East Coast (blue), b) West Coast (red), c) Wales (green) and d) English Channel (grey). Missing haplotypes are shown as empty dots and the numbers in the branches are the sites where the changes are present.

Two phylogenetic trees were constructed using parsimony and Bayesian analyses. In the parsimony tree (fig. 3) all the haplotypes present in East Scotland (blue) were clustered together along with the haplotypes SW2007/84 exclusive to the English Channel (orange) and the haplotype SW2006/98 which was present in Wales and the English Channel. Two haplotypes of the Mediterranean were also present in this cluster that is supported with a high bootstrap value (73). The Bayesian tree (Fig. 4) supports the same cluster with a very high posterior probability (91).

The exclusive haplotypes from the West Coast (red), one from the English Channel and one from Wales are all part of a polytomy with very low bootstrap support (58) in the parsimony tree (fig. 3). In the Bayesian tree (fig. 4) four of the West Coast haplotypes, one of the English Channel and one of Wales were part of clusters that comprise sequences from Portugal, Mediterranean and Black Sea, supported with a 0.84 posterior probability. The remaining West Coast haplotypes were part of another cluster supported with a 0.84 posterior probability that includes also sequences from Portugal and Mediterranean Sea.

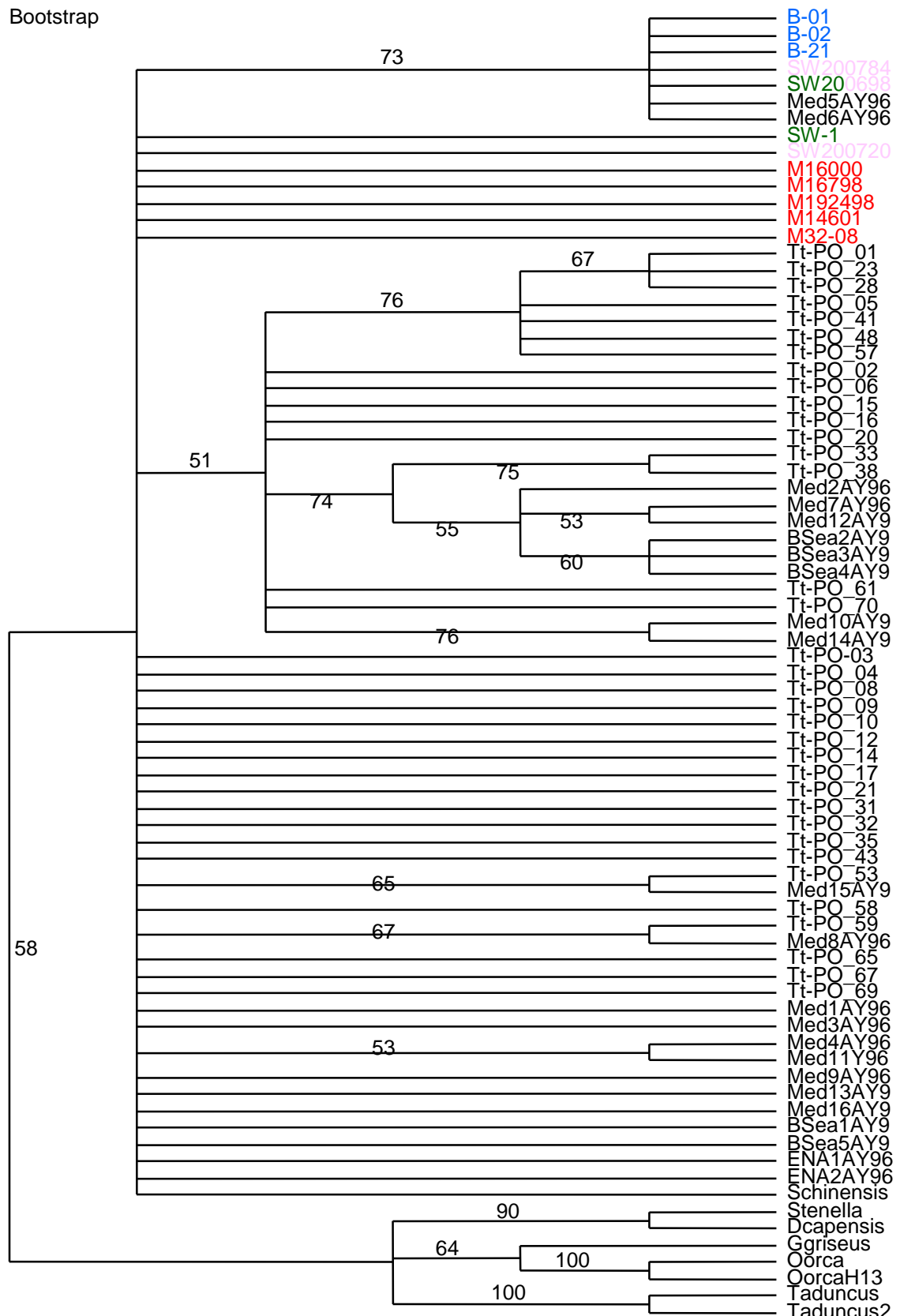


Figure 3. Consensus Parsimony tree with 1000 bootstrap replicates. The outgroups were: *Stenella* spp, *D. capensis*, *G. griseus*, *O. orca* (2 haplotypes) and *T. aduncus* (2 haplotypes). Haplotypes from this study are shown in colors representing where they came from East Coast (blue), West Coast (red), Wales (green) and English Channel (pink).

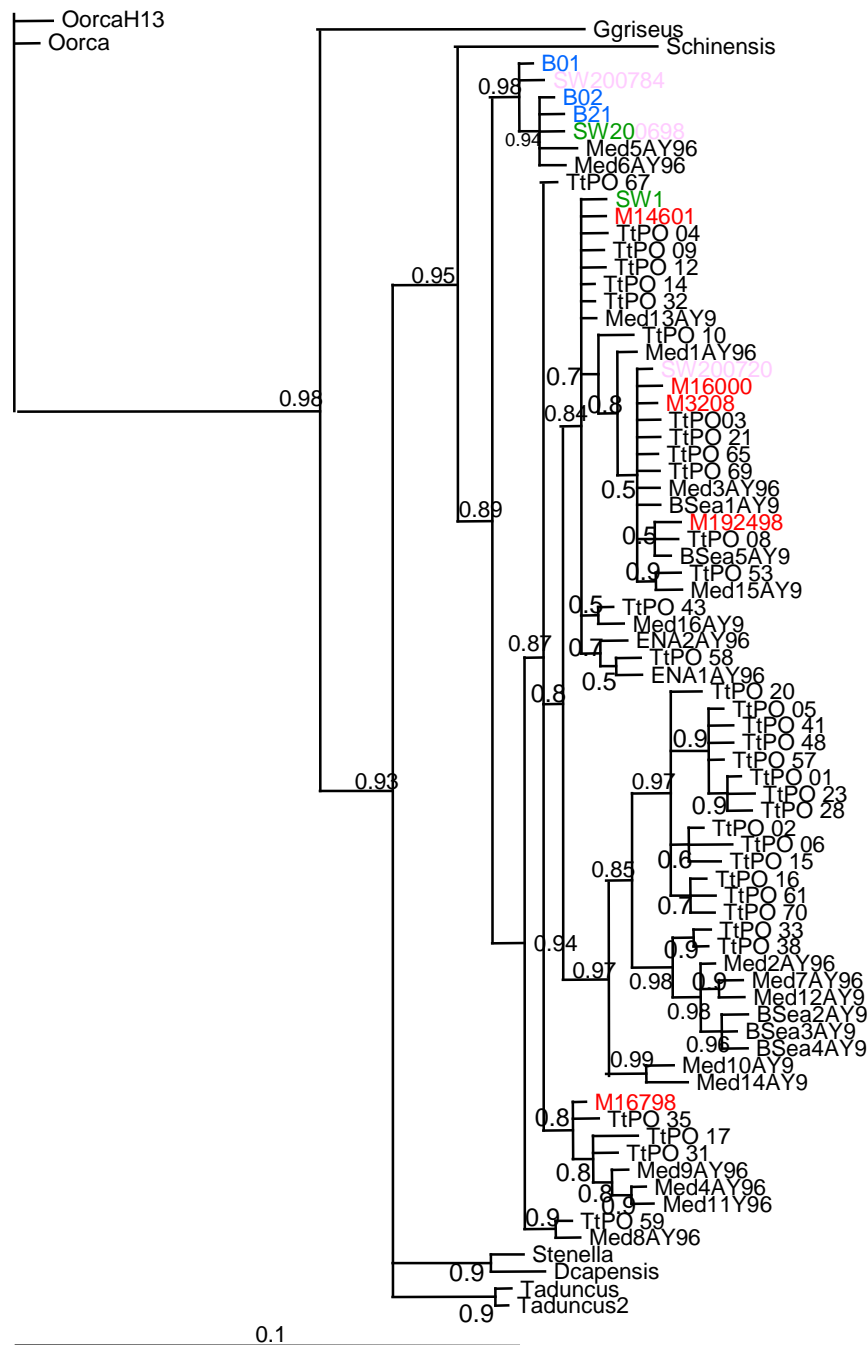


Figure 4. Consensus Bayesian tree showing posterior probabilities. Two haplotypes of *O. orca* were used as an outgroup. Haplotypes from this study are shown in colors representing where they came from East Coast (blue), West Coast (red), Wales (green) and English Channel (pink).

2.3.2. Microsatellites

2.3.2.1. Genetic diversity

Of twenty microsatellites originally selected, 17 amplified successfully using multiplex conditions. Table 7 shows the percentage of error for each locus in both strandings and biopsies. Locus D22 shows over 20% of error for both biopsies and strandings. Loci TV5, MK9 and Dde84 show over 20% only for strandings but not for biopsies and locus Dde 70 shows over 20% only for biopsies. In LG1 a total of 10 biopsied individuals and 17 strandings were repeated representing 23.8% of total biopsies and 24.63 % of total strandings. In LG2 a total of 13 biopsied individuals and 30 strandings were repeated, representing 30.95% of the total biopsies and 43.47% of total strandings. Finally LG3 repeated 5 biopsied individuals that represent 11.9% of the total biopsies and 24 stranded individuals that represent 34.78 % of total strandings. Locus Dde70 remained in the dataset because the sample size to calculate the error rate for biopsies in LG3 was only $n=11$ and due to the nature of the error rate scoring this values seems to be inflated.

The 17 loci were analysed in 110 individuals for linkage disequilibrium and three pairs resulted with highly significant *p-values* for the test. These were (Tur61/Dde70), (D22/Dde84) and (Dde84 and Dde72). All the loci were also tested for Hardy-Weinberg deviations and if they were out of equilibrium after Bonferroni correction in more than one population (Table 8) they were eliminated from the analysis. Finally, Microchecker's results show that Locus EV1 showed the presence of null alleles and an excess of heterozygotes. Locus Tur91 also showed null alleles and locus Dde84 showed null alleles and excess of homozygotes as well as the presence of stuttering bands that might have caused mistakes while genotyping. As a result of all theses tests, locus Tur91, Dde84, EV1 and D22 were eliminated from further analyses and the

data was analyzed with 13 loci. Details of genotype scoring process are shown in Appendix C.

Table 7. Genotyping error in biopsies and strandings. A total of 42 biopsies and 69 strandings were analyzed. In LG1 biopsies were repeated in average 2.6 times and strandings 2.5 times. In LG2 biopsies were repeated in average 2.46 times and strandings 2.7 times and LG3 repeated biopsies in average 2.2 times and strandings 2.5 times.

Locus	Locus Group	Motif repeated	Error Biopsies	Error Strandings
D08	LG1	TG	0.05	0.1
D22	LG1	(CA)-TA-(CA)	0.24	0.297
TV7	LG1	CA	0.05	0.094
TV5	LG1	CA	0	0.222
MK6	LG1	GT	0	0.053
MK8	LG2	GT	0.088	0.049
EV1	LG2	(AC)(TC)	0.087	0.167
MK9	LG2	CA	0	0.225
Tur117	LG2	GATA	0	0.03
Tur91	LG2	GATA	0	0.15
Tur48	LG2	GATA	0	0.08
Dde61	LG3	CTAT	0.083	0.035
Dde70	LG3	CA	0.2727	0.155
Tur138	LG3	GATA	0.0909	0.019
Tur105	LG3	GATA	0	0
Dde84	LG3	CA	0	0.212
Dde72	LG3	CTAT	0.091	0.1

Genetic diversity values such as expected (H_e) and observed Heterozygosity (H_o), number of alleles per population and allelic richness were obtained for the 13 loci (Table 7). After Bonferroni correction non-significant p-values for the Hardy-Weinberg test should be larger than 0.00096. Only three loci showed significant values: MK8, Dde70 and Tur117 (shown in bold). The loci were out of Hardy-Weinberg equilibrium only for the West Coast of Scotland

(MK8 and Dde70) and Wales (Tur117), for this reason they were kept in the analysis (Table 7).

Overall low heterozygosity values are present in all the populations except for the English Channel individuals that showed the highest values. Allelic richness values were also low across all populations except the English Channel but with the West Coast of Scotland showing 2 loci with highest values around 4.0 (Dde 70 and Dde72) (Table 7).

Table 8. Genetic diversity in nuclear microsatellites for all the populations. Sample size (N). For each locus: total number of alleles (n), Observed (*Ho*) and expected (*He*) heterozygosity, number of alleles (n) and allelic richness (A). Loci out of equilibrium are shown in bold.

Loci	East Coast (N=63)	West Coast (N=19)	Wales (N=12)	English Channel (N=7)
D08 (n=9)	(n=3) A=2.2031 He=0.4623 Ho=0.4603 P= 0.6106	(n=5) A=2.252 He=0.3294 Ho=0.2632 P=0.2049	(n=4) A=2.235 He=0.2990 Ho=0.1667 P=0.1191	(n=5) A=3.393 He=0.7253 Ho=0.5714 P=0.1793
TV7 (n=11)	(n=4) A=2.005 He=0.4549 Ho=0.4444 P=1.0000	(n=8) A= 2.601 He=0.4075 Ho=0.2632 P=0.0198	(n=4) A= 2.235 He=0.2990 Ho=0.2500 P=0.3179	(n=5) A= 3.393 He=0.7253 Ho=0.7143 P=0.4953
TV5 (n=7)	(n=4) A=2.254 He=0.5133 Ho=0.5397 P=0.0295	(n=5) A= 3.036 He=0.5245 Ho=0.4737 P=0.3568	(n=3) A= 2.000 He=0.2278 Ho=0.2500 P=1.000	(n=7) A= 4.335 He=0.8571 Ho=0.7143 P=0.1664
MK6 (n=14)	(n=6) A= 2.964 He=0.6697 Ho=0.6349 P=0.052	(n=8) A= 3.682 He=0.6553 Ho=0.5789 P=0.0263	(n=5) A= 3.396 He=0.5637 Ho=0.5833 P=0.8308	(n=8) A= 4.791 He=0.9121 Ho=0.7143 P=0.1956
MK8 (n=9)	(n=6) A= 3.216 He=0.7148 Ho=0.6984 P=0.2735	(n=6) A= 3.007 He=0.6316 Ho=0.3158 P=0.0000	(n=4) A= 2.494 He=0.5254 Ho=0.5000 P=0.0726	(n=6) A= 4.060 He=0.8352 Ho=0.8571 P=0.6612
MK9 (n=7)	(n=4) A= 1.734 He=0.2840 Ho=0.2698 P=0.0144	(n=6) A= 2.794 He=0.5761 Ho=0.3158 P=0.0035	(n=3) A= 1.847 He=0.3007 Ho=0.2500 P=0.2602	(n=3) A= 2.774 He=0.6703 Ho=0.4286 P=0.3247
Tur 117	(n=3) A=1.536 He=0.1888 Ho=0.1746 P=0.0133	(n=3) A=2.509 He=0.5439 Ho=0.4211 P=0.3341	(n=2) A=1.333 He=0.0833 Ho=0.0833 P=0.0000	(n=3) A=2.359 He=0.5385 Ho=0.2857 P=1.071
Tur48 (n=6)	(n=5) A= 3.072 He=0.6749 Ho=0.8095 P=0.34	(n=4) A= 2.799 He=0.5694 Ho=0.4211 P=0.183	(n=4) A= 3.355 He=0.6984 Ho=0.5833 P=0.062	(n=5) A= 3.494 He=0.7692 Ho=0.7143 P=0.7263
Dde61 (n=7)	(n=7) A=2.771 He=0.6478 Ho=0.6667 P=0.1455	(n=7) A= 3.91 He=0.7321 Ho=0.6316 P=0.0156	(n=2) A= 1.962 He=0.4891 Ho=0.4167 P=1.000	(n=5) A= 3.895 He=0.8242 Ho=0.7143 P=0.3627
Dde70 (n=14)	(n=10) A=3.883 He=0.8028 Ho=0.8571 P=0.2304	(n=13) A=4.477 He=0.8720 Ho=0.6316 P=0.0000	(n=7) A= 3.857 He=0.8152 Ho=0.7500 P=0.7637	(n=7) A= 4.901 He=0.9231 Ho=1.0000 P=0.5497
Tur 138 (n=6)	(n=4) A=2.046 He=0.3989 Ho=0.3810 P=0.2056	(n=5) A= 3.029 He=0.6421 Ho=0.6842 P=0.5564	(n=3) A= 1.500 He=0.1630 Ho=0.0833 P=0.0421	(n=5) A= 3.647 He=0.8022 Ho=0.8571 P=0.2336
Tur 105 (n=4)	(n=3) A= 2.746 He=0.5420 Ho=0.4762 P=0.5112	(n=3) A= 2.75 He=0.1429 Ho=0.1053 P=1.0000	(n=4) A= 3.464 He=0.2500 Ho=0.2500 P=0.3154	(n=2) A= 2.000 He=0.2286 Ho=0.2857 P=1.0000
Dde72 (n=9)	(n=7) A= 2.124 He=0.3845 Ho=0.4286 P=0.0104	(n=8) A= 4.165 He=0.6715 Ho=0.4737 P=0.0191	(n=5) A= 3.639 He=0.7302 Ho=0.5833 P=0.0702	(n=8) A= 4.611 He=0.8901 Ho=0.7143 P=0.3298

2.3.2.2. Population Structure: Three different methods to estimate Population differentiation

Pairwise population differentiation indices F_{ST} , Rho_{ST} and D_{EST} were calculated for all populations (Table 8). Significant values were in all comparisons of the East Coast with other locations. Population differentiation was stronger between the East Coast of Scotland and the English Channel. Other pairwise comparisons were not significant, possibly due to the reduced sample sizes of these populations compared to the East Coast.

Table 8. Population differentiation between pairwise populations with microsatellites. Significant scores are in bold and the p-value is shown below them. Lower diagonal shows F_{st} values (P -values were obtained after 6000 permutations). Average variance components of Rho_{ST} shown in the upper diagonal along with Jost's (2008) D_{EST} shown in [].

Population	East Coast n=64	West Coast n=18	Wales n=12	English Channel n=7
East Coast	-	0.0547 p=0.00100 [0.0568]	0.0897 p=0.00200 [0.0422]	0.1119 p=0.00200 [0.1184]
West Coast	0.0956 p=0.00017	-	0.0473 p=0.09500 [0.0116]	0.0209 p=0.34000 [0.0067]
Wales	0.0781 p=0.00017	0.0149 p=0.43967	-	0.1487 p=0.02100 [-0.1963]
English Channel	0.0957 p=0.0005	0.0228 p=0.48483	0.0879 p=0.25900	-

2.3.2.2. Bayesian clustering assignment of populations (The problem of determining K)

Structure was run with the number of populations set from 1 to 10. Evanno et al. (2005) recommendations were followed using the admixture model with correlated frequencies among populations. This model is recommended when populations are likely to have a common ancestor.

In fig. 5 the average value of $\text{LnP}(D)$ for each value of K simulated was plotted. The results show a plateau, but it is not clear whether the plateau starts at $K=3$ or $K=4$. The difference in the average of the log-likelihood values between $K=3$ and $K=4$ is around 30. Pritchard et al. (2007) suggests that when the differences are so small, the true value of K is more likely to be the smaller of the two.

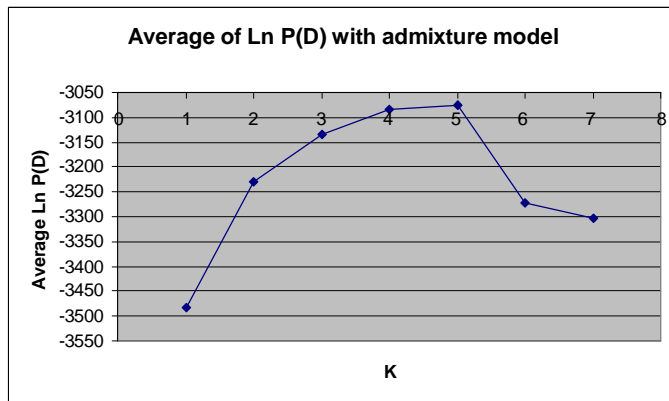


Figure 5. Average value of the $\text{LnP}(D)$ of the posterior probability for ten runs of each K for Admixture Model.

Following the Evanno et al. (2005) procedure for deciding the true value of K , I calculated the rate of change between the different values of K . This detects the value of K for the uppermost level population structure for the populations tested (fig. 6). This method identifies $K=2$ as the number of subgroups.

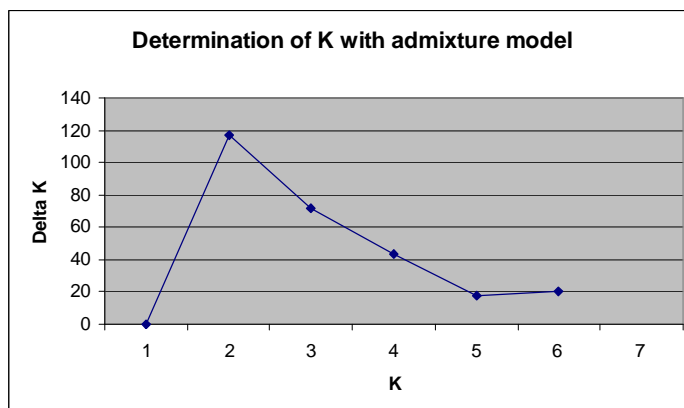


Figure 6. Graphical representation of ΔK calculated as: $\Delta K = |L''(K)| / \text{SD}[L(K)]$. This graph is the last step in Evanno's et al. (2005) procedure to determine the true K in Structure 2.2.

The 'No admixture model' was also tested with our data. Structure recommends this model for populations that are fully discrete and it is meant to be better at detecting subtle structure. This model assumes that all the individuals come from just one population. The average $\text{LnP}(D)$ for the posterior probabilities is shown in fig. The start of the plateau is not clear either $K=3$ or $K=4$.

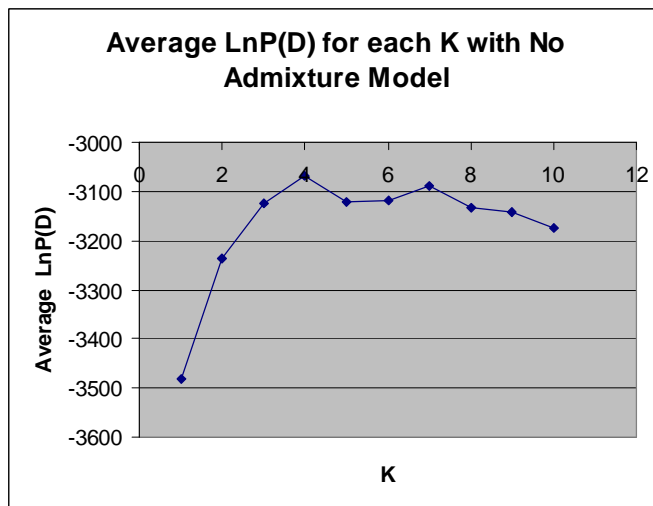


Figure 7. Average value of the Ln of the posterior probability for ten runs of each K for 'No admixture model'.

The Evanno et al. (2005) procedure again identified the maximum value of K at $K=2$ and we can observe another peak of ΔK at $K=4$ but much lower.

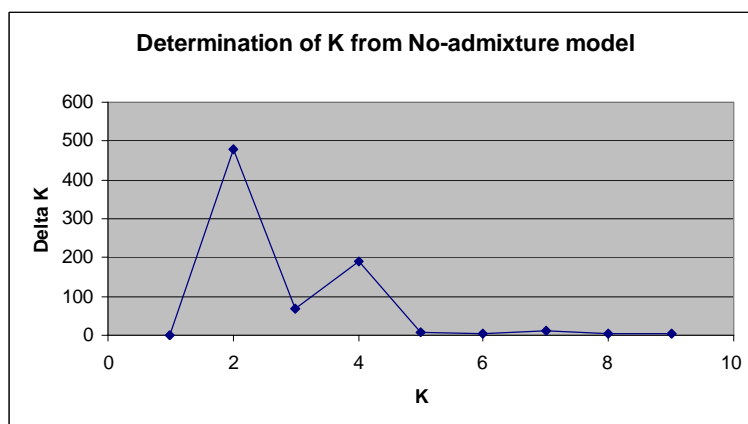


Figure 8. Graphical representation of ΔK calculated as: $\Delta K = |L''(K)| / \text{SD}[L(K)]$. This graph is the last step in Evanno et al.. (2005) procedure to determine the true K in Structure 2.2.

These results give us 2 different scenarios. $K=4$ implies the East Coast of Scotland is isolated from the rest of the neighbouring populations, which is consistent with the F_{st} results. A connection between a part of the West Coast of Scotland that includes the Barra biopsied individuals is connected with Wales. The other part of the West Coast is clustered with the English Channel animals (fig. 9).

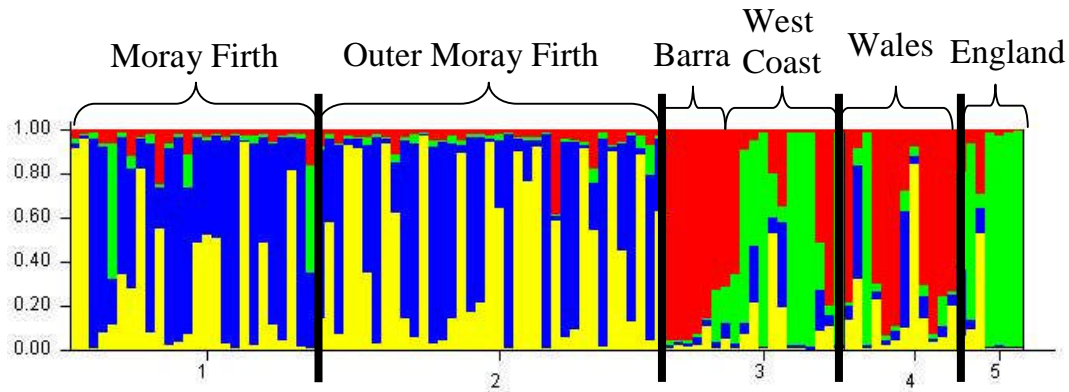


Figure 9. Barplot of the likelihood (Y-axis) of each individual's (X-axis) assignment to a particular population for $K=4$. Pop 1= East Coast of Scotland (Moray Firth), Pop 2= East Coast of Scotland Outer Community, 3) West Coast of Scotland, Pop 4= Wales and Pop 5= English Channel.

When the Evanno et al. (2005) procedure is applied we obtain $K=2$ and the scenario suggests a connection between the East Coast of Scotland, part of the West Coast of Scotland and most of Wales. On the other hand the rest of the West Coast of Scotland and England (fig. 10).

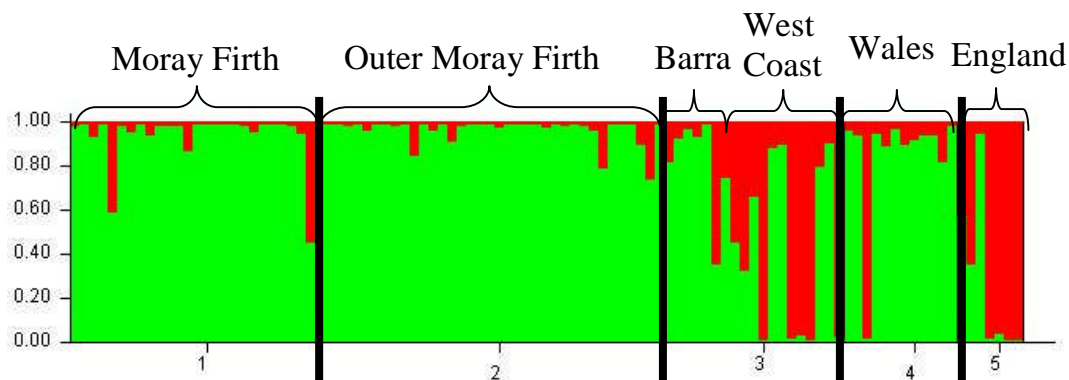


Figure 10. Barplot of the likelihood (Y-axis) of each individual's (X-axis) assignment to a particular population for $K=2$. Pop 1= East Coast of Scotland (Moray Firth), Pop 2= East Coast of Scotland Outer Community, 3) West Coast of Scotland, Pop 4= Wales and Pop 5= English Channel.

The scenario with $K=3$ maintains a deep division in the West Coast of Scotland into 2 groups, Barra and the rest of the West Coast. The Barra individuals grouped with Wales while the rest of the West Coast has a connection with the population from English Channel. In this scenario the East Coast of Scotland is not isolated and a proportion of individuals belong to the same cluster as the Barra-Wales individuals.

The main problem with the different K 's is the establishment of gene flow between the East Coast of Scotland, Wales and Barra. It is clear in all the possible outcomes of K , that there is a connection between the West Coast of Scotland population of Barra and Wales. The individuals from the English Channel and the rest of the West Coast of Scotland are also consistent throughout all the scenarios.

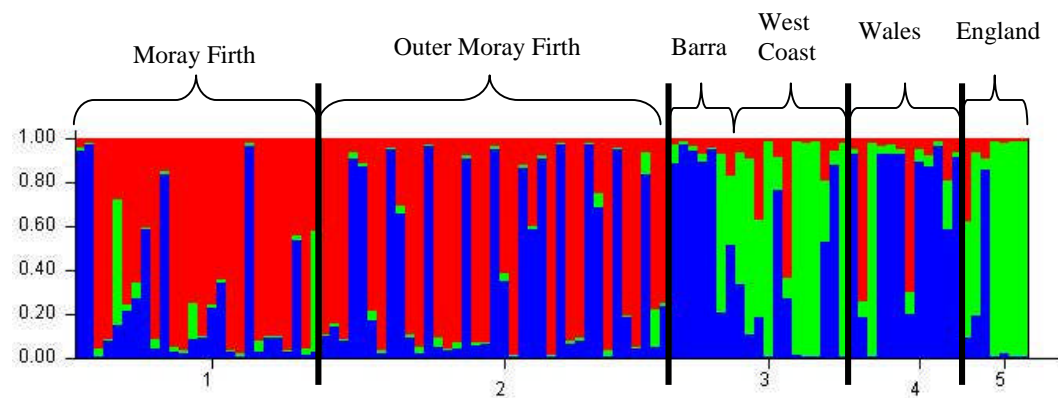


Figure 11. Barplot of the likelihood (Y-axis) of each individual's (X-axis) assignment to a particular population for $K=3$. Pop 1= East Coast of Scotland (Moray Firth), Pop 2= East Coast of Scotland Outer Community, 3) West Coast of Scotland, Pop 4= Wales and Pop 5= English Channel

Results for only the East Coast samples with the admixture model do not include the Evanno method as it fails to acknowledge only one population. The average of the $\text{LnP}(D)$ for each K is shown in fig. 12. The best values are for $K=2$ and $K=3$, with very similar likelihoods, -1540.8 and -1539.6 respectively.

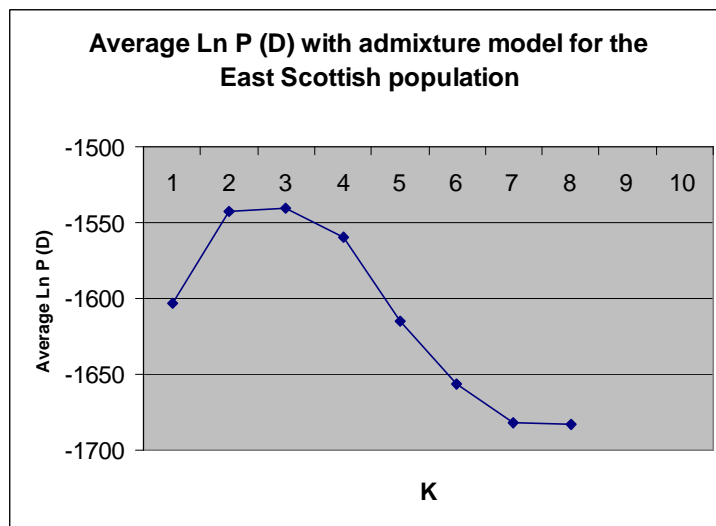


Figure 12. Average value of the LnP(D) of the posterior probability for eight runs of each K for Admixture Model.

For $K=2$ the analyzed individuals have almost a 50% probability to belong to either of the 2 populations (Table 9). When $K=3$ the probabilities for each inferred cluster are even lower. The barplots in fig. 13 show the graphic representation for both scenarios $K=2$ and $K=3$ where there is no obvious distinction between strandings and biopsies. The only safe conclusion is that the samples come from one population.

Table 9. The probabilities of assigning individuals to a particular population given a particular value of K

Given K	Inferred Clusters				No of Individuals
2		1	2		63
		0.470	0.530		
3		1	2	3	63
		0.381	0.357	0.263	

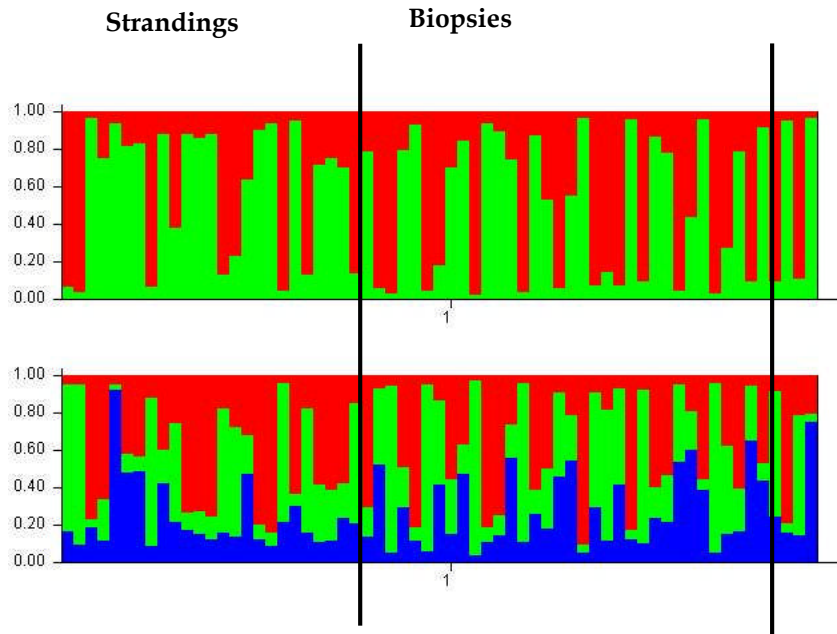


Figure 13. Barplots of the East Scottish population. The upper barplot represents to $K=2$ and the lower barplot to $K=3$. The biopsied individuals are shown within the black lines and the last 4 individuals are also strandings.

2.3.2.3. Determination of migration rates and sex-biased dispersal

To clarify the ambiguity in determining the true value of K , the mean migration rates along with 95% confidence intervals were calculated with the program BayesAss 1.3 (Table 11). This program calculates the proportion of individuals that do not migrate, described as the migration rate into the same population (diagonal shown in bold). It also calculates the migration rates from each population into another.

BayesAss 1.3 simulates migration rates and the correspondent confidence intervals for a dataset with no information on migration. This procedure gives us a mean migration rate and a confidence interval of values that are expected merely by chance. The migration rates obtained with the real data should be different to the rates and confidence intervals simulated with the same number of populations. In a scenario with 4 populations the mean migration rate for non migrants (diagonal) in a non informative data set is

0.833 and the confidence interval is [0.675, 0.992]. All our values for non-migrants are significantly different from chance (Table 11).

For the 4 populations, the mean migration rate should be different from 0.0553 and the confidence should not overlap with the following interval [0.000155, 0.218]. Unfortunately the values of migration rates, from one population into another, fall within this non-significant interval (Table 11). This means that the sample size from the neighboring populations of the East Coast of Scotland is not sufficient to accurately calculate migration rates.

Table 11. Mean migration rates of individuals into populations along with 95% confidence intervals are shown. The proportion of non-migrant for each population is shown in the diagonal in bold.

	From East Coast	From West Coast	From Wales	From English Channel
Into East Coast	0.989723 (0.973044, 0.999036)	0.00252248 (5.53204e-06, 0.0116464)	0.00537869 (5.83124e-05, 0.0169869)	0.00237576 (1.78402e-05, 0.0111435)
Into West Coast	0.0349544 (0.000744548, 0.101667)	0.719411 (0.670332, 0.801117)	0.210383 (0.12052, 0.28838)	0.035252 (0.000494114, 0.105662)
Into Wales	0.156355 (0.0413052, 0.256361)	0.0480826 (0.0015044, 0.121274)	0.775839 (0.688092, 0.898503)	0.0197231 (0.000183089, 0.0836096)
Into English Channel	0.0854917 (0.0103915, 0.196245)	0.103126 (0.00371304, 0.23127)	0.0773185 (0.00442729, 0.19703)	0.734064 (0.668808, 0.864282)

Sex-biased dispersal was calculated with FSTAT for all the populations, with a one tailed test. Fst for females was 0.0808 and Fst for males was 0.1143 with a non-significant *p-value* of 0.73.

2.3.2.5. Relatedness between populations

Average relatedness values between the individuals of each population were obtained (Table 12). The West Coast of Scotland was divided in the 2 subunits detected by Structure 2.2: 'Barra' and 'rest of the West Coast'. The Barra subpopulation shows relatedness values typical of parent-offspring or full-siblings. Wales shows relatedness value similar to half-siblings, followed by the East Coast of Scotland. The rest of the West Coast of Scotland and the English Channel individuals show negative values of relatedness, which means that the individuals of those groups are less related than what expected.

A distance matrix was obtained by subtracting 1 from the pairwise relatedness scores for all the individuals analyzed. The distance matrix obtained was used to construct a UPGMA tree to visualize the relationships between the individuals of the 4 populations (Fig. 12 and 13). The complete tree is shown in Appendix E. In this tree the East Coast of Scotland has no close relationship with individuals from the other populations except from a small group of individuals from Wales (cluster 6, fig.13).

This figure also shows the intricate relationship between the 'Barra' animals and a portion of the Welsh population (cluster 4, fig 12). The clear division between the individuals from the West Coast of Scotland (except Barra) and the English Channel individuals is also observed (cluster 1, fig. 12), this cluster is a sister group to the rest of the clusters.

One individual from the English Channel strandings is clustered with the East Coast individuals (cluster 7, fig. 13). This individual has the predominant mitochondrial haplotype from the East Coast of Scotland. These findings could be either evidence of migration of individuals from the East Coast or misplacement of samples due to the strong currents.

Table 12. Relatedness among populations calculated with 13 loci. Standard error after 100 jackknife simulations.

Population	Relatedness	Standard error
East Coast	0.1663	0.0367
Barra	0.4879	0.1444
West Coat rest	-0.1302	0.0348
Wales	0.2084	0.0816
England	-0.1411	0.0354

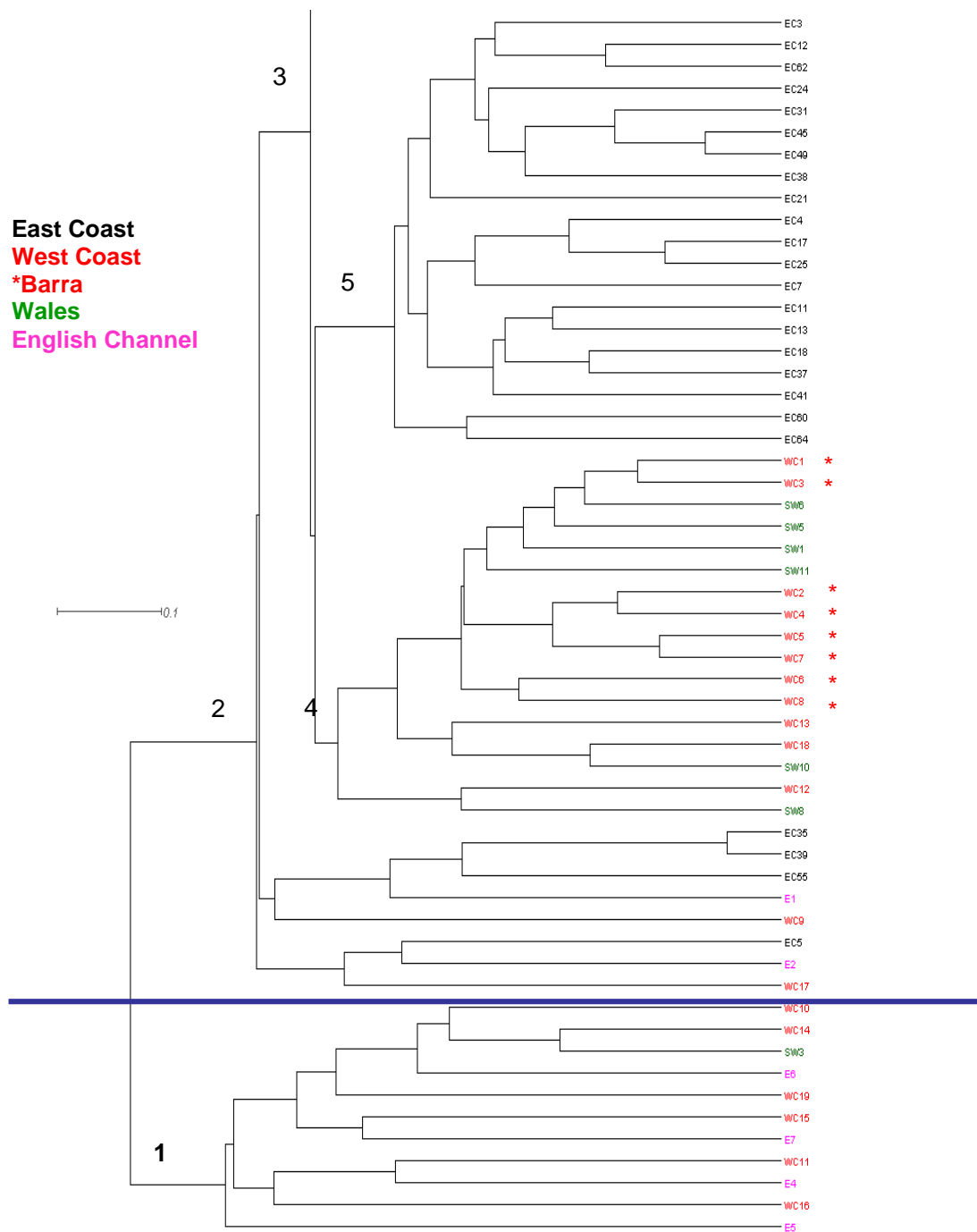


Figure 12. Lower half of a UPGMA tree showing the distances between individuals according to the Queller and Goodnight (1987) index of relatedness. Cluster 1 is divided by a blue line. This cluster appears as a sister group to the rest of the individuals analyzed. Biopsied individuals from Barra are marked with *.

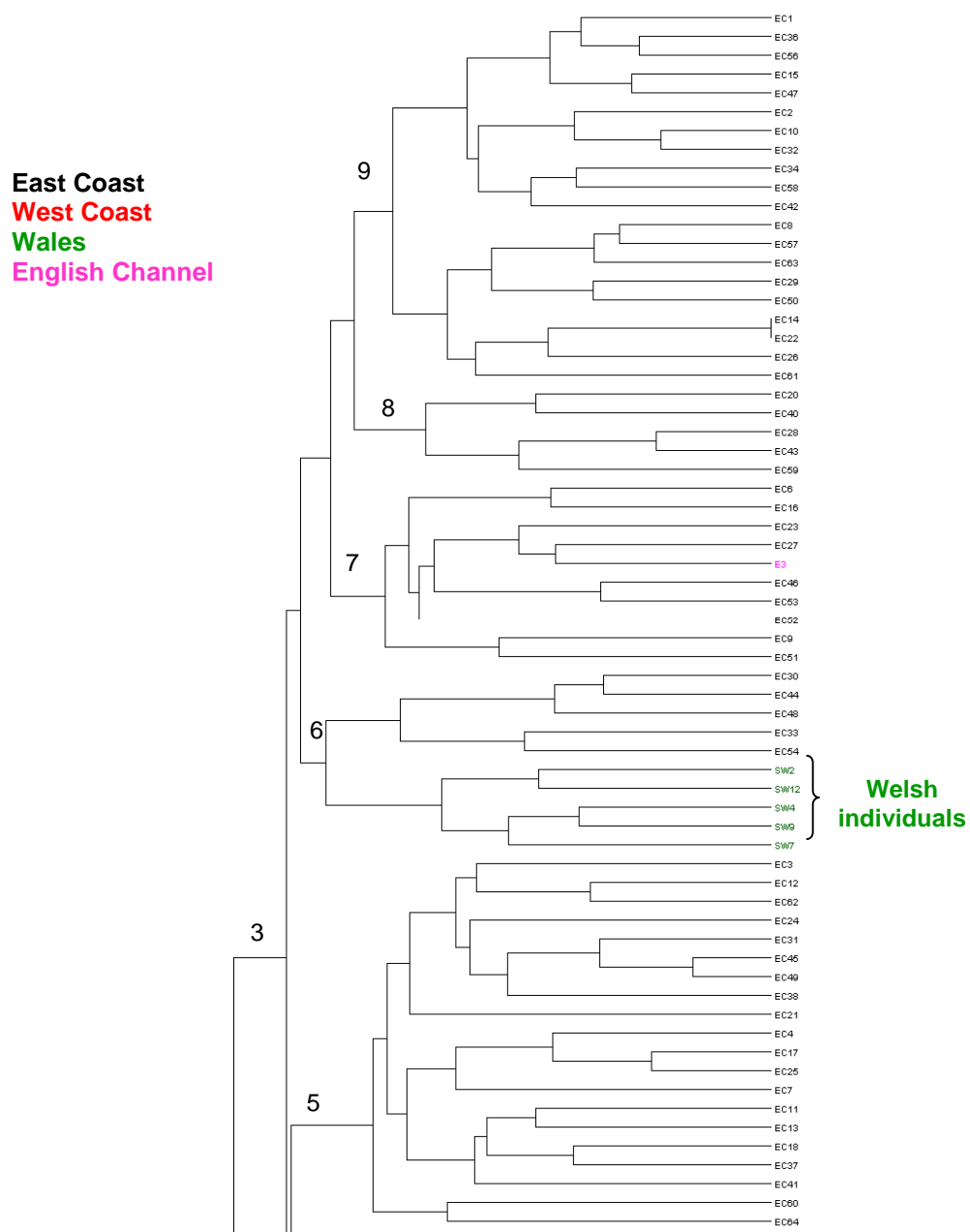


Figure 13. Continuation of the UPGMA tree showing the distances between individuals according to the Queller and Goodnight (1987) index of relatedness.

2.4. Discussion:

2.4.1. Genetic diversity

Previous studies of the UK bottlenose dolphins have found low values of genetic diversity along the East Coast of Scotland with evidence that the population is isolated and possibly locally adapted (Nichols et al. 2007; Parsons et al. 2002). These studies used a limited set of genetic markers and only stranding samples. The implications for the use of strandings in the population structure patterns observed in cetaceans have not yet been fully explored. It is possible that the patterns could be an artefact of the small sample size, misplacement of the samples due to the currents or a lack of definition from the molecular markers. When comparing the error rate for each locus between strandings and biopsies, I found that for 12 of the 17 loci analyzed the error rate is higher in strandings than in biopsies, but as mentioned before the method used could potentially overestimate the error rate. Future work should test the significance in this difference as well as analyzing the factors contributing in it (e.g. quality sample, motif repeated, number of repetitions of the PCR reaction, etc).

Thirty-five biopsy samples were taken from the East Coast of Scotland (Table 1). Considering that previous estimates of the population size were around 129 individuals (Quick 2006; Wilson et al. 1999 ; Wilson et al. 1997a), this represents 27% of the population. The 'Barra' population in the West Coast of Scotland is believed to be approximately 15 individuals (Grellier & Wilson 2003), and seven samples were taken (Table 1), this would represent almost 50% of this population.

After the sexing of the samples it was revealed that a higher number of males were biopsied in the East Scottish population. The sex difference in biopsy sampling has been reported in other genetic studies (Quérrouil et al. 2009)

presumably because females actively avoid boats, especially if they have small calves. In my study I have concentrated on the better marked individuals to avoid double sampling. The sex bias in our samples could be explained if males are better marked than females since they tend to be more aggressive with each other. In the West Coast of Scotland population of Barra, six out of seven samples were females. This already suggests either a very different social structure or a different behaviour towards the boat in the two populations.

In the first genetic study of the dolphins of the Scottish East Coast, Parsons et al. (2002) analyzed only 29 stranded individuals from five populations. In a 549 bp section of the mitochondrial control region, they found 21 polymorphic sites and only eight haplotypes: 2 haplotypes from the East Coast of Scotland, 3 haplotypes from the West Coast of Scotland, 3 haplotypes from Wales, 1 from the English Channel and 4 from Ireland. We analyzed 87 individuals for 507 sites of the same section of the control region and found 12 haplotypes in the same populations except Ireland; 3 haplotypes in the East Coast of Scotland, 3 haplotypes in Wales, 5 haplotypes in the English Channel and 7 haplotypes on the West Coast of Scotland. The mtDNA results confirmed the exceptionally low genetic diversity of the East Coast of Scotland ($h=0.3500$, $\pi=0.000747$) in 56 samples analyzed, very similar to what Parsons et al. (2002), ($h=0.476$, $\pi=0.0009$) found for the 15 samples they had for the same region. Natoli et al. (2004) found a slightly higher value while pooling all UK populations into an Eastern North Atlantic group, $h=0.42$ and $\pi=0.016$. These values are still low compared to other populations worldwide; e.g. Mediterranean Sea $h=0.94$, $\pi=0.023$; Western North Atlantic Pelagic $h=0.88$, $\pi=0.022$, Gulf of Mexico $h=0.72$, $\pi=0.013$, Chinese Pelagic $h=0.92$, $\pi=0.024$ (Natoli et al. 2004). Other populations show much higher levels of genetic diversity as found in the Azores and Portugal ($h=0.957$, $\pi=0.015$); Madeira ($h=0.927$, $\pi=0.012$) and in the mainland of Portugal ($h=0.857$, $\pi=0.014$) (Qu  rouil et al. 2007).

The remaining populations within the UK in order of increasing genetic diversity are: Wales ($h=0.5556$, $\pi=0.005479$), West Coast of Scotland ($h=0.6250$, $\pi=0.007495$) and the English Channel ($h=0.9333$, $\pi=0.008284$). The scores for the East Coast of Scotland resembled those of Western North Atlantic coastal bottlenose dolphins ($h=0.43$, $\pi=0.018$) (Hoelzel et al. 1998b; Natoli et al. 2004); and overall coastal populations of Little Bahama Bank in Bahamas ($h=0.763$, $\pi=0.0066$) (Parsons et al. 2006).

In microsatellite genetic diversity, the heterozygosity values for the 13 alleles from the East Coast, West Coast and Wales, had very similar values but the samples from the English Channel had higher values. A similar pattern was found by Natoli et al. (2004) with similar values of heterozygosity among several coastal populations worldwide but much higher values in the pelagic populations of the North Atlantic. These higher values of heterozygosity were also found among populations from the Azores that are not significantly different from the pelagic populations of the North Atlantic (Qu  rouil et al. 2007).

Nichols et al. (2007) analysed stranding samples from the main populations of the UK waters with 5 microsatellites. The main objective of their study was to establish the origin of an extinct population of bottlenose dolphins in Flixborough, Northeast England. They grouped the samples in the following populations: Flixborough, East Coast of Scotland and Outer UK (comprising: West Coast of Scotland, English Channel and Wales). For the three loci in common with their study, we found very similar values of allelic richness between both studies. For the East Scottish population: D08 allelic richness $AR=2.203$ and 3 alleles, while they found $AR=2.994$ and 3 alleles, for MK8 they found $AR=4.950$ and 5 alleles and we found $AR=3.217$ and 6 alleles.

For the OUK (Outer UK) group Nichols et al. (2007) found higher values in comparison to the ones I found for each population. They mentioned that the

extremely high genetic diversity of the OUK group could be due to sampling several populations. This can be better observed when we compare their OUK with the single populations in this study. For D08 they obtained: AR=6.620 and 8 alleles; while this study reported AR=2.131 (5 alleles) for the West Coast, Wales AR=2.235 (4 alleles) and the English Channel AR=3.393 (5 alleles). For locus MK8 they obtained AR=6.299 and 7 alleles, while for the West Coast AR=2.911 (6 alleles), Wales AR=2.494 (4 alleles) and the English Channel AR=4.060 (6 alleles).

2.4.2. Phylogeography

The haplotype network displays this very strong pattern of differentiation between the East and the West Coast of Scotland graphically, but it also shows that the main haplotype present in the East Coast of Scotland is present in smaller numbers in the other three populations, mostly in Wales. This confirms Parsons et al. (2002) previous suggestion, that there is a higher mitochondrial gene flow pattern between the East Coast of Scotland and Wales than between the East and West Coast of Scotland.

There also was a higher diversity of haplotypes on the West Coast of Scotland as was suggested by the large presence of missing haplotypes (empty circles) representing individuals that were not sampled. One haplotype from Wales and one from the English Channel are connected to the West Coast haplotypes. It is important to notice that all the 'Barra' individuals as well as three strandings showed the same haplotype (M160/00). Two of three strandings come from the Western Isles, which are part of the Outer Hebrides, and the other one from the Isle of Mull. This suggests that the 'Barra' individuals consists of one matriline, as most of the biopsy samples that show this haplotype came from better marked individuals, which are possibly the oldest individuals in the group.

Both the parsimony and Bayesian trees (Fig. 3 and Fig. 4) cluster East Scotland haplotypes with samples from the West Coast of Scotland, Wales and the Mediterranean with high support values (73 bootstrap and 0.98 posterior probability). These relationships were shown previously by Natoli et al. (2005), where Scottish haplotypes also clustered with several individuals of the Eastern North Atlantic and Eastern Mediterranean.

The West Coast of Scotland haplotypes along with one haplotype from Wales and one from England clustered together with haplotypes mostly from the Azores (Qu  rouil et al. 2007), as well as some haplotypes from the Mediterranean and Baltic Sea (Natoli et al. 2004, 2005) with a posterior probability of 0.84 in the Bayesian tree. The relationships of these haplotypes in the parsimony tree are not resolved and they show a very low support (bootstrap 58). Qu  rouil et al. (2007) found a lack of genetic structure among bottlenose dolphin populations from the Azores. They suggested that the lack of structure was caused by the presence of pelagic individuals that have very large ranges and have shown to maintain high levels of gene flow. This suggests that the West Coast of Scotland origins might be related to WNAP (Western North Atlantic Pelagic) populations and it explains the high contrast in genetic diversity between the East and the West Coast of Scotland. Tezanos-Pinto et al. (2009) compared mitochondrial DNA haplotypes world wide and they found that the WNA pelagic haplotypes were spread worldwide, suggesting a lack of habitat specificity.

2.4.3. Population differentiation

For the mitochondrial DNA we calculated both population differentiation indexes (F_{st} and φ_{st}) with a strong significant differentiation between all the pairwise comparisons. The strongest differentiation found was between the East and West Coasts of Scotland ($F_{st}=0.52794$ vs $\varphi_{st}=0.85958$). The West

Coast of Scotland also showed significant pairwise comparison scores with Wales ($F_{st}=0.37874$ and $\varphi_{st}=0.60348$) and the English Channel ($F_{st}=0.22723$ and $\varphi_{st}=0.51472$). There was also a significant value between the English Channel and East Scotland ($F_{st}=0.21290$ and $\varphi_{st}=0.27106$). The smallest F_{st} score was found between East Scotland and Wales ($\varphi_{st}=0.15003$). It was observed that all the φ_{st} values were bigger than the F_{st} values in all pairwise comparisons, which suggests that these strong differences were due to a founder effect or a restricted gene flow in the past.

These results contrast with the much lower population differentiation value ($\varphi_{st}=0.145$) found by Nichols et al. (2007) comparing the East Coast of Scotland (NES) and the Outer UK (West Coast of Scotland, Wales and English Channel). These differences could be due to pooling several populations together as one population.

When the population differentiation index F_{ST} was measured with microsatellites, significant pairwise comparisons were found only between the East Coast of Scotland and the other populations. These values were similar to the ($F_{ST}=0.049$) pairwise comparison between NES and OUK in Nichols et al. (2007) study. This value suggests a fine scale structure with limited gene flow but not such a severe restriction of gene flow as found between the Western North Atlantic (WNAC) populations and OUK with a $F_{ST}=0.224$ score (Nichols et al. 2007). Population differentiation indices suggest that the East Coast of Scotland have different allele frequencies than the rest of the populations and that gene flow between them is restricted.

It has been suggested that the rejection of panmixia given by significant values of F_{st} is not enough to determine population structure and assign management units (Palsboll et al. 2007; Taylor & Dizon 1999). It has also been

shown that F_{ST} values are constrained towards higher levels of genetic diversity and due to these limits they underestimate population differentiation when heterozygosity is very high (Jost 2008). Pairwise comparisons for Rho_{ST} and D_{EST} (Jost's alternative measure of differentiation) showed very similar values and they were higher for the F_{ST} values except for the East and West Coast comparison. Rho_{ST} comparison between Wales and the English Channel was the highest significant value reported for this estimate ($Rho_{ST} = 0.1487$ p -value 0.021) but was very different from the D_{EST} estimation (-0.1963). D_{EST} estimates however seem to have the same problems as G_{ST} (and analogues) in obtaining consistent values of differentiation when mutation rate is high, but D_{EST} is particularly affected when migration is included in the model, two very important factors in natural populations (Ryman & Leimar 2009). We are comparing populations that have very different levels of heterozygosity (English Channel has considerably higher heterozygosity than the other populations), which raises concerns about the correct measure of differentiation that should be employed in these studies. It makes sense to calculate all three measures and compare them; looking at the discrepancies and similarities and taking in account the levels of heterozygosity of each population. This proves challenging for bottlenose dolphin populations (*Tursiops truncatus*) that have two different ecotypes (pelagic and coastal) with very different values of genetic diversity and overlapping ranges (Hoelzel et al. 1998b; Natoli et al. 2004).

2.4.4. Bayesian clustering assignment of populations

If we accept STRUCTURE identification of 4 populations following (Pritchard, 2009) the East Coast of Scotland appears isolated from the neighboring populations, just as the population differentiation indices suggest. This was previously suggested by Nichols et al. (2007) and Natoli et al. (2005) and concerns about its viability were raised.

However, if the scenario is $K=3$, then the results show that the East Coast of Scotland is divided in 2 groups of individuals and that there is connectivity between a proportion of the East Coast of Scotland, the West Coast population of Barra and Wales. Lusseau et al. (2006) suggested that the East Scottish population of bottlenose dolphins is subdivided into two groups of individuals, one resident in the Moray Firth and another that moves along the range of the population and which I have called the Outer community. All the biopsied individuals were sampled near St. Andrews; thus they should constitute the outer community, but it is not known if they were the same individuals that Lusseau et al. (2006) referred to. Structure showed very little evidence of any structure within the East Coast population or between biopsies and strandings, the values of K with the highest maximum likelihoods ($K=2$ and $K=3$) showed very low probabilities of the animals belonging to any of the populations exclusively.

When we look at the individual level, we can see that most of the individuals from the East Coast of Scotland that belonged to the East Coast-West Coast-Wales cluster were biopsied males ($n=12$). This could suggest male biased dispersal, or it could be an artefact of biopsy sampling that was biased towards males. Natoli et al. (2005) suggested a higher rate of female emigration from the East Coast of Scotland than immigration to it based on mtDNA gene flow, but they did not find significant evidence for sex-biased dispersal with microsatellites. Unfortunately, the small sample sizes of the West Coast of Scotland, Wales and English Channel populations made it impossible to obtain significant results on present migration rates or sex biased dispersal with FSTAT.

When following the Evanno et al. (2005) procedure a clear peak in $K=2$ was found. This scenario suggests a connection between the East Coast of Scotland, Barra and Wales on one hand and on the other the English Channel individuals with the rest of the West Coast of Scotland. This procedure

obtains the 'uppermost hierarchical level of structure', which in bottlenose dolphins populations could mean the differentiation between Coastal and Pelagic individuals.

In all possible outcomes of K (2, 3 and 4) the division of the West Coast of Scotland into 2 subunits is always present. The connection between Barra and Wales, as well as the connection between the rest of the West Coast of Scotland and the individuals from the English Channel is also consistent.

2.4.5. Relatedness between populations

The relatedness values between populations showed that, the only 2 groups with negative values of relatedness were the English Channel and the rest of the West Coast of Scotland. These samples formed a cluster that is the sister group to all other bottlenose dolphins in the UPGMA tree. These same strandings from the English Channel were previously analyzed by (Natoli et al. 2005). In her study these samples were clustered by STRUCTURE in the Eastern North Atlantic population that comprised Portugal and Galicia. This suggests the important influx of pelagic individuals along the West Coast of Scotland and the restricted genetic flow between pelagic and coastal populations, like the one found in the Western North Atlantic (Hoelzel et al. 1998b).

The highest values of relatedness were assigned to the biopsied individuals of Barra, suggesting that this group of individuals are a mitochondrial matriline and very close relatives. The Welsh population also showed high values of relatedness and the UPGMA tree revealed tight connections between Barra and a proportion of individuals from Wales.

Scenarios for $K=3$ and $K=2$ suggest that the East Coast Scottish population of bottlenose dolphins is not completely isolated from the neighbouring populations in the UK. The method suggested for determining the value of K by Pritchard et al. (2000) is not straight-forward. While he recommended the correlated frequencies model, he also highlights that it might overestimate K , for this reason it is recommended to select the smallest value of K . These methods might struggle to find a structure in populations with low F_{st} values (Hubisz et al. 2009) similar to the ones found in this study.

Unfortunately a direct measurement of the migration rates between the populations was not possible due to the small sample sizes of the populations except for the East Coast of Scotland. The UPGMA tree showed a small group of Welsh dolphins closely related to the East Scottish ones. This might be evidence of a low level of gene flow between these two populations or it could also be due to a common ancestor between the 2 populations since some of the Welsh individuals have Hap1 which is the predominant one in the East Coast of Scotland.

The East Coast of Scotland showed very low values of genetic diversity both with mitochondrial and microsatellite markers as was previously found by Parsons et al. (2002), Nichols et al. (2007) and Natoli et al. (2004, 2005).

However, all previous studies had small sample sizes that were only from strandings. Due to this reduced genetic diversity, there are concerns about the isolation of the East Scottish population from the other populations of the UK waters. Nichols et al. (2007) suggested that the dynamics in UK populations meet some criteria of meta-population (dependence on local habitat patches and restricted gene flow) and that the East Scottish population show signs of decline.

Natoli et al. (2005) and Möller et al. (2007) showed that local habitat dependence is important for bottlenose dolphins and constitutes an important factor promoting genetic structure. My results revealed that the dynamics of population structure in the populations of bottlenose dolphins around the UK waters resemble those of Pelagic and Coastal populations in the Western North Atlantic (Hoelzel et al. 1998b). The West Coast of the UK is clearly divided into sympatric populations, some constituted by highly related individuals (Barra and Wales) and the other by individuals that are not (English Channel and rest of the West Coast of Scotland).

Concerns about the viability of the Barra population need to be raised. This population is only approximately 15 individuals all in one matriline. Its mitochondrial origin appears to resemble a pelagic population similar to the ones in the Azores. Despite the strong connection between Barra and Wales, the meta-population dynamic indicates that these small and specialized subpopulations could easily go extinct. It is important to investigate if this subset of 15 individuals constitutes an independent population, or if it is a migrating group from a bigger population.

Despite the presence of significant population structure calculated with F_{st} , some scenarios of STRUCTURE establish a connection between the East and West Coast of Scotland and Wales but these results were not clear.

Relatedness analysis show a small proportion of Welsh individuals highly related to East Scottish ones, if this is not an effect of common ancestry it could be an evidence of a small amount of gene flow. Only recently photo-identification efforts have confirmed the presence of East Scottish individuals in the West Coast of Scotland (Robinson et al. 2009) but this cannot yet be detected by molecular markers. This could be due to the individuals migrating but not reproducing in other populations, to a lack of resolution of the markers due to small sample size or to a very recent migration of individuals that cannot be picked up by genetic markers yet. We must

remember that records of bottlenose dolphins were not common until the end of the 1800s. If this is indeed the case, recent gene flow between the populations is not likely to be detected yet.

Chapter 3. Association and relatedness in the East Scottish population of bottlenose dolphins

3.1. Introduction:

Tinbergen (1953) defined the term 'social', in the simplest way, by describing a situation that comprises more than one individual. This definition not only refers to a relationship with a passive aggregation of two or more individuals, but to a reaction or interaction with each other. It is obvious how being part of a group confers benefits to an individual; they can forage in a more efficient way and protect each other from predators (Tinbergen 1953). How does this social organization arise and evolve? How do individuals constitute and organise these groups, and deal with environmental pressures? These were some of the questions addressed by Tinbergen, whose studies may give us an insight into the importance of social structure in individuals, populations and species.

To address these questions, Hinde (1976) proposed a framework in which the study of the social structure of a population should not only be based on the aggregation of individuals, but in the quality, content and temporal pattern of their interactions. Whilst recording these interactions the observer could be biased towards the most obvious or impressive individuals or situations, and for this reason it is important to measure these interactions in a quantitative way (Cairns & Schwager 1987). The most common and basic measure for these interactions is given by association indices (Whitehead 1997), and among studies of cetacean social structure, the association index developed by Cairns & Schwager's (1987) is the most widely used.

To reliably estimate association in a reliable way with this index there are several considerations to take into account:

- 1) An accurate definition of a group and its members, which reflects the complexities of the studied individuals.
- 2) A suitable sampling period that avoids autocorrelation and is independent of ongoing/previous behaviours.
- 3) Random sampling of individuals forming dyads.

Bottlenose dolphins live in fission-fusion societies (Connor et al. 2000a; Wells 1991; Wells et al. 1980). In these societies group membership changes frequently and individuals are associated with different individuals at different times (Cairns & Schwager 1987). Despite this characteristic long term associations of bottlenose dolphins have been documented in some well studied populations since the 1970s (Scott et al. 1990; Wursig & Harris 1990; Wursig & Wursig 1977).

One of the best studied populations of bottlenose dolphins is in Sarasota Bay, Florida. Surveys have been carried out for over 30 years revealing long term associations based on age and gender (Scott et al. 1990). Similar strong, long-lasting associations have also been found off the coast of Argentina in a group of 53 animals that have been studied for 8-12 years (Wursig and Harris, 1991). These long lasting associations have also been found in Australia (Connor et al. 2000b; Smolker et al. 1992), in the Indian River Lagoon in Florida (Kent et al. 2008) and in shorter time periods in Bahamas (Parsons et al. 2003) and Port Stephens (Moller et al. 2001, 2006) among others.

Patterns and strength of association may vary between both sexes and age classes. In Sarasota Bay adult and sub-adult males are rarely seen together (Scott et al. 1990). Sub-adult males form large bachelor groups and adult males form small, long-lasting groups that move between female groups.

Females form bands of individuals that have calves of similar ages or no calves at all.

In Shark Bay, Australia, Smolker et al. (1992) also found stable association patterns that differed between genders. Females in this population form larger social networks but less stable associations than males. This same pattern is also present in the Indian River Lagoon in Florida, where individuals associate preferentially in small groups of the same sex (Kent et al. 2008).

Common strong associations are usually observed for mother-calf pairs. These relationships show high association coefficients across several species of cetaceans; for example Atlantic spotted dolphins (*Stenella frontalis*) in the Bahamas (Welsh & Herzog 2008), sperm whales (*Physeter macrocephalus*) (Gero et al. 2005) and bottlenose dolphins (*Tursiops spp*) (Grellier et al. 2003; Grellier & Wilson 2003; Kent et al. 2008; Scott et al. 1990; Shane 1986) among others.

Possibly the most remarkable association pattern is the one displayed by male alliances in bottlenose dolphins. These alliances have been documented in at least four populations: Shark Bay, Australia (Connor et al. 1992), Port Stephens, Australia (Moller et al. 2001), Sarasota Bay, Florida (Wells, 1986 cited in: Duffield and Wells, 2002) and Little Bahama Bank, Bahamas (Parsons et al. 2003), with alliance males showing association coefficients similar to mother-calf pairs (Connor et al. 1992, 1996, 2001). Strong association coefficients between males were also found in some dyads of the Indian River Lagoon in Florida but they were not as common as in known 'alliances' populations (Kent et al. 2008).

Not all delphinid species show strong long lasting associations; marine Tucuxi dolphins, (*Sotalia guianensis*), in Southeastern Brazil show few strong

associations between individuals (Santos & Rosso 2008). This fluid social structure pattern has also been documented in bottlenose dolphins off Point Lookout, Queensland, Australia (Chilvers & Corkeron 2002) and in the Moray Firth, Scotland (Wilson 1995).

When studying the social structure of a population the idea of strong associations being kin related is immediately considered. Kin selection theory states that individuals gain 'inclusive fitness' through the reproduction of related individuals as well as through their own reproduction (Hamilton 1963; Maynard-Smith, 1964). This idea supports behaviours such as altruism, aggression, cooperation, selfishness and spite, but it does not explain competition between relatives (Griffin & West 2002) or cooperation between non-related individuals and the mechanisms that maintain them (Clutton-Brock 2009).

The concept of reciprocal altruism was first described by Trivers in 1971; he proposed that individuals of different species (or same species but not kin) exchange assistance or resources in order to gain a service in the future, by those who have been helped (Trivers 1971). It can also happen that the gain obtained by working together in a group of unrelated individuals is large enough that it exceeds the costs invested in cooperation. This is called 'group augmentation' (Kokko et al. 2001). If a member of a group has been assisted by another member of the group that is not necessarily its kin, it can respond positively to this behaviour by also cooperating within this group. If this behaviour becomes common, the group will reach a stable state of 'generalized reciprocity' no matter who they are cooperating with (Pfeiffer et al. 2005). Finally a dominant individual can 'manipulate' other individuals in the group to obtain benefits from them by means of fear and harassment (Clutton-Brock & Parker 1995). In some animals both related or non-related males cooperate to increase the reproductive success of both partners (Huck et al. 2005)

Recent studies suggest that the importance of kin selection is overestimated and sometimes confounded by high levels of relatedness shown by individuals interacting in a particular task (Griffin & West 2002).

This makes us wonder if the formation of male alliances and female bands in bottlenose dolphins is explained by kin selection or by non-kin cooperative mechanisms. Recent studies show a mixed pattern. First order male alliances in Shark Bay (Krutzen et al. 2003) and Little Bahama Bank (Parsons et al. 2003) are formed by related individuals, while alliances in Sarasota Bay (Duffield and Wells 2002) and Port Stephens (Moller et al. 2001) are not. The same has been shown for female bands in Sarasota Bay that are composed of individuals from several matriline (Wells & Duffield 2002). While in Port Stephens females seem to associate preferentially with their kin primarily when they share the same reproductive state (Moller et al. 2006).

In this study I investigated the patterns of association and relatedness of the East Scottish population of bottlenose dolphins to contribute information to this question. The East Coast of Scotland population occupies the northernmost range of the distribution of the species (Wilson 1995) and appears to be isolated and locally adapted (Nichols et al. 2007). The description of mechanisms underlying the social structure of delphinids, is a crucial piece of information in understanding the evolutionary history of social structure in delphinids and cetaceans.

3.2. Methods:

3.2.1. Photo-identification:

Individual identification of bottlenose dolphins through nicks and notches on the dorsal fins has been shown to be a reliable and commonly used method in ecological and behavioural studies since the 1970s (Wursig & Wursig 1977). In this study photo-identification of bottlenose dolphins in St. Andrews Bay was carried out during May-September in 2006 and 2007. Surveys were conducted from a polyethylene RIB style boat (5.7 meters) with a four-stroke outboard engine (100 horse power). To increase sample size, high quality photo-identification data, taken by Quick (2006) were incorporated. These data were obtained from focal follows and opportunistic encounters with dolphins, during 35 separate days from July to September in 2003 and 2004.

Trips were conducted between Arbroath and Fife Ness when sea state was between Beaufort 0-3 with winds of less than 10 mph and it was dry. Every trip was divided into several 'encounters' throughout the day. An encounter was defined as an individual or groups of individuals that were photographed during a period of time (Bejder et al. 1998, following Slooten et al. 1993). Encounters were up to 60 min long. If a group changed composition more than 50% (50% of its members left), it was considered a new encounter. Data on weather conditions, sea state, GPS position, group size and behaviour were recorded in a 'St Andrews area Encounter Sheet' (Appendix A).

Photographs were taken with a Canon EOS 30D camera, and a Sigma 100-300 mm, F4 zoom lens. It was attempted that both sides of the dorsal fin would be photographed for all the individuals in a group during each encounter.

3.2.2. Biopsy sampling

Once a high quality photograph was obtained from each individual within the group, a target individual was chosen for biopsy sampling. Target individuals were chosen on the basis of obvious marks on the dorsal fin that would aid immediate recognition to avoid double sampling. After a target individual was chosen and it was confirmed that a high quality photograph of it was available a biopsy attempt was made. With every attempt, a series of photographs were taken to record the biopsy shot and confirm the identity of the biopsied animal.

Biopsies were taken with a PAXARMS 745 biopsy system with a red dot scope. The boat was positioned parallel to the target individual and at least two surfacings were observed before shooting the target individual. We tried to assure that there were no other animals swimming close to the target animal. The sampling was mainly focused on well-marked adults but it was also attempted to sample some calves that were older than a year. Krutzen et al. (2002) found that the biopsies were smaller or unsuccessful when they hit the dorsal fin or the immediate area surrounding it, for this reason the red dot was directed towards (approximately 5-10 cm) the area surrounding the base of the dorsal fin as recommended by the authors.

Information was recorded on every biopsy attempt, whether it was successful or unsuccessful. The individual catalogue number or characteristic marks, as well as detailed information about the shot (position, side of the fin, angle to the boat, etc) was recorded in a 'St Andrews area Genetic Biopsying' sheet (Appendix A).

The behavioural reaction to all biopsy attempts, along with the behaviour shown five minutes after the shot were also recorded. The dart was retrieved from the water with a net. If the biopsy was successful the dart containing it

was kept in aluminium foil in a cool box with frozen packages. The dart was sealed and marked with masking tape and the time of the shot was noted. The dart number and time of the shot were also recorded in the biopsy sampling sheet (Appendix A). Photographs of the biopsy wound were taken whenever the biopsied animal was found in subsequent encounters to monitor the condition of the wound, according to previous studies (Krützen et al. 2002). Reports of biopsy sampling reaction and wound healing rates are shown in Appendix B.

3.2.3. Association analysis:

Only high quality photographs of well marked individuals were used for the 'Association' analysis. Calves or animals with no obvious marks were excluded. Quality grading of the photographs was completed following Quick's (2006) classification (Appendix A). Only photographs that were marked as grade 3.1, 3.2 or 3.3 of this classification were analyzed.

To obtain a quantitative measure of the level of associations between the individuals of the population, the Half Weight Index (HWI) was calculated with the software SOCPROG 2.3 (Whitehead 1997). To identify significant levels of association between dyads, the permutation test suggested by Bejder et al. (1998) and implemented in SOCPROG 2.3 was applied permuting association values within samples. Tests were two tailed ($\alpha=0.05$) and 5 tests were run with 5000, 10000, 15000 and 20 000 permutations, to see when the p-value of the test stabilized. To avoid autocorrelation bias, sampling was restricted to a daily basis (Smolker et al. 1992; Karczmarsky et al. 2005; Chilvers & Corkeron 2002; Santos & Rosso 2008) and only individuals that have been seen at least 5 days during the study period were included.

3.2.4. Sexing:

To determine the sex of the individuals a PCR co-amplification of ZFX and SRY genes was carried out following Rosel (2003) with some modifications. PCR reactions were prepared with a Multiplex PCR kit from (QIAGEN). Ten μ l PCR reactions included 5 μ l of multiplex mix, 3 μ l of primer mix and 2 μ l of DNA (10ng).

Primer concentration was 10 pM of ZFX0582F and ZFX0923R and 3 pM of TtSRYR and PMSRYF. PCR started with 15 min of denaturation at 95 °C to activate the HotStart Taq polymerase of the Multiplex kit, followed by 30 cycles of 30 sec at 94 °C, 90 sec at 51 °C and 45 sec at 72 °C, final extension was carried out for 2 min at 72 °C. The PCR products were visualized with UV light in a 2% agarose gel dyed with Ethidium Bromide.

3.2.5. Relatedness:

Seventeen previously reported polymorphic nuclear microsatellites were analyzed for 101 individuals from the 4 putative populations described in Chapter 2: East Coast of Scotland, West Coast of Scotland, Wales and English Channel. The original source of the microsatellites and the PCR details are shown in Table 1 (Appendix C). The seventeen microsatellites were amplified with a fluorescent dye to be able to read them in an automatic sequencer (Beckman Coulter). The markers were divided in 3 loci groups that were amplified with a Multiplex PCR kit from (QIAGEN) with conditions shown in Table 1. Details on the procedure to score the alleles from each locus are shown in Appendix C.

Table 1. Multiplex PCR Locus Groups Characteristics. For each Locus Group (LG) the following information is listed: Locus name, concentration of primers pairs (F/R), type of dye and concentration of dye are shown.

LG1

Locus	F/R[μ M]	DYE	[DYE]	Locus	F/R[μ M]	DYE	[DYE]
TexVet5	2 μ M	D4	0.12 μ M	Tur4_80	2 μ M	D4	0.16 μ M
TexVet7	2 μ M	D3	0.8 μ M	MK9	2 μ M	D2	0.8 μ M
D08	2 μ M	D3	0.6 μ M	EV1	2 μ M	D3	0.8 μ M
D22	2 μ M	D4	0.12 μ M	Tur_91	2 μ M	D4	0.16 μ M
MK6	2 μ M	D2	0.8 μ M	Tur_117	2 μ M	D4	0.16 μ M
				MK8	2 μ M	D4	0.08 μ M

LG3

Locus	F/R[μ M]	DYE	[DYE]
Tur105	2 μ M	D3	0.8 μ M
Dde72	2 μ M	D4	0.16 μ M
Tur138	2 μ M	D3	0.8 μ M
Dde84	2 μ M	D4	0.16 μ M
Dde70	2 μ M	D3	0.8 μ M
Dde61	2 μ M	D2	0.8 μ M

PCR reactions consisted of 10-20 ng of genomic DNA, 5 μ l of Multiplex Mix and 3 μ l of primer mix. PCR profile was as following: 95°C for 15 min followed by 30 cycles of 94°C for 30sec, 60°C for 90 sec and 71°C for 45sec, with a final extension of 72°C for 2 min.

DNA was extracted twice for approximately 10% of the individuals from each population analyzed to control for errors in sample labelling and identity. PCR reactions for approximately 30% of the individuals were repeated at least 1 more time and sometimes up to 6 times as described in Chapter 1. Only one mother-calf pair was obtained from stranded samples and it was used to calibrate allele sharing between pairs of individuals in each loci.

Linkage disequilibrium and Hardy-Weinberg deviations were tested with GENEPOP v. 3.1d (Raymond & Rousset 1995a) and null alleles were investigated with Microchecker. After these tests only 13 microsatellites were chosen for the analyses, details are shown in Chapter 2. A rarefaction test was

performed with RE-RAT (<http://people.musc.edu/~schwacjh/>) to determine if the number of loci employed in this study was enough to accurately determine relatedness in this population. The rarefaction analysis does simulations to observe how the relatedness estimations change while more loci are added.

Van de Castele et al. (2001) compared the performance of 3 relatedness estimators and showed that their performance was affected by different factors like population composition, sampling variance, number of loci and number of alleles. They suggested to test what they called the 'best estimators', separately when obtaining pairwise comparisons of relatedness in a population. In this study we compared 2 of the 3 'best estimators' suggested by them, Queller and Goodnight (1989) and Lynch and Ritland (1999).

Several assessments of relatedness were carried out due to the nature of the complex relationships between the UK populations of bottlenose dolphins. If relatedness is calculated for populations that have very different origins, these differences will exaggerate the relatedness values within each population. As suggested in Chapter 2, the English Channel samples and almost half of the West Coast samples seem to have a very different origin (possibly pelagic) from the Welsh and East Scottish samples. For this reason average relatedness and male/female average relatedness were calculated only for the East Scottish population. To obtain uncertainty values of both estimates of R, Re-RAT calculated them with a jackknife over loci of 100 simulations.

Queller and Goodnight general formula is:

$$\hat{r}_{xy} = \frac{\sum_l \sum_a (p_{y_a} - p^*)}{\sum_l \sum_a (p_{x_a} - p^*)}$$

Where:

r_{xy} = relatedness between individual x and y

l = indexes loci

α = indexes allelic position

P_x = frequency (0.5 or 1) of the allele at allelic position α in individual x

P_y = frequency (0.0, 0.5 or 1) of the allele at allelic position α in individual y

P^* = population frequency of the allele under consideration

Lynch and Ritland formula is:

$$\hat{r}_{xy}(l) = \frac{p_a(S_{bc} + S_{bd}) + p_b(S_{ac} + S_{ad}) - 4p_a p_b}{(1 + S_{ab})(p_a + p_b) - 4p_a p_b}$$

Where:

a and b = allelic position 1 and 2 of individual x

c and d = allelic position 1 and 2 of individual y

$S_{ab} = 1$ if individual x is homozygous

$S_{ab} = 0$ if individual is heterozygous

$S_{ac} = 1$ if allele a from individual x is the same as allele c from individual y

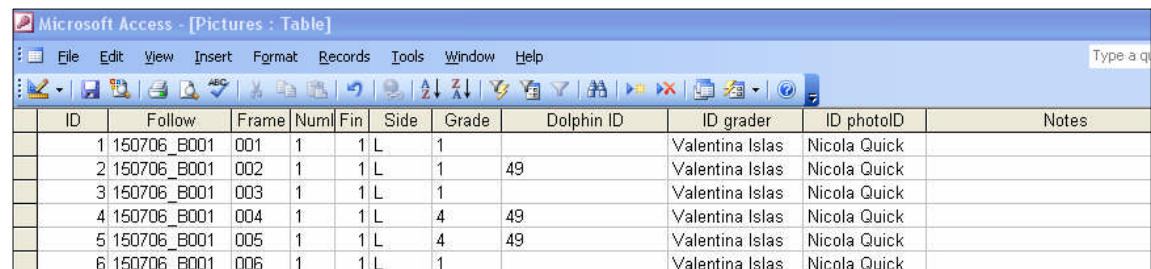
$S_{ac} = 0$ if otherwise

Finally a matrix of mtDNA haplotypes was created for the biopsied individuals to assess the relationship between maternal lineages and association patterns. If two individuals shared the same haplotypes it was score as 1 and if they did not share it was scored as 0. To compare the mtDNA matrix and the association matrix a Mantel test was performed with the statistical program R with 1000 permutations. The same test was performed to compare the association matrix with the relatedness matrix obtained with RE-RAT.

3.3. Results:

3.3.1. Photo-identification

In 38 trips bottlenose dolphins were encountered on 98 occasions. A total of 12 726 photographs were taken during the field seasons of 2006 and 2007. Photographs were renamed to contain the date and number of the trip (e.g. 240807_B38). A Microsoft Access (2003ver) database was created to organize and mark the quality of the material. An entry in the database was created for each fin in every photograph (fig 1).



The screenshot shows a Microsoft Access window titled 'Microsoft Access - [Pictures : Table]'. The window contains a table with the following data:

ID	Follow	Frame	Numl	Fin	Side	Grade	Dolphin ID	ID grader	ID photoID	Notes
1	150706_B001	001	1	1	L	1		Valentina Islas	Nicola Quick	
2	150706_B001	002	1	1	L	1	49	Valentina Islas	Nicola Quick	
3	150706_B001	003	1	1	L	1		Valentina Islas	Nicola Quick	
4	150706_B001	004	1	1	L	4	49	Valentina Islas	Nicola Quick	
5	150706_B001	005	1	1	L	4	49	Valentina Islas	Nicola Quick	
6	150706_B001	006	1	1	L	1		Valentina Islas	Nicola Quick	

Figure 1. Section of the photo-identification database. Fore each photograph, details on the number of fins, the side of the fin photographed, the dolphin identity (ID) along with the name of the person that graded and identified the photograph are included.

3.3.2. Association analysis

Over the four years included in the analysis (2003, 2004, 2006 and 2007) a total of 61 trips and 182 encounters were recorded. From these efforts a total of 138 well-marked individuals were identified and included in the analysis. After restricting to individuals that had been seen on more than five days over the four years, we obtained association coefficients for 63 individuals, of which 19 were molecularly confirmed males and seven were females. Previous studies have calculated the population size of East Scotland to be approximately 129 individuals (Wilson 1997; Wilson 1999 and Quick 2006), which means that we covered approximately 48% of the population in this association study.

The distributions of the Half Weight Index (HWI) association indices are shown in figure 2. It can be observed in the histogram that the predominant association value is between 0-0.2. The highest value in the daily restricted analysis is 0.82. A matrix with the pairwise association indices for all the individuals after the 5 days sampling restriction is shown in figure 3.

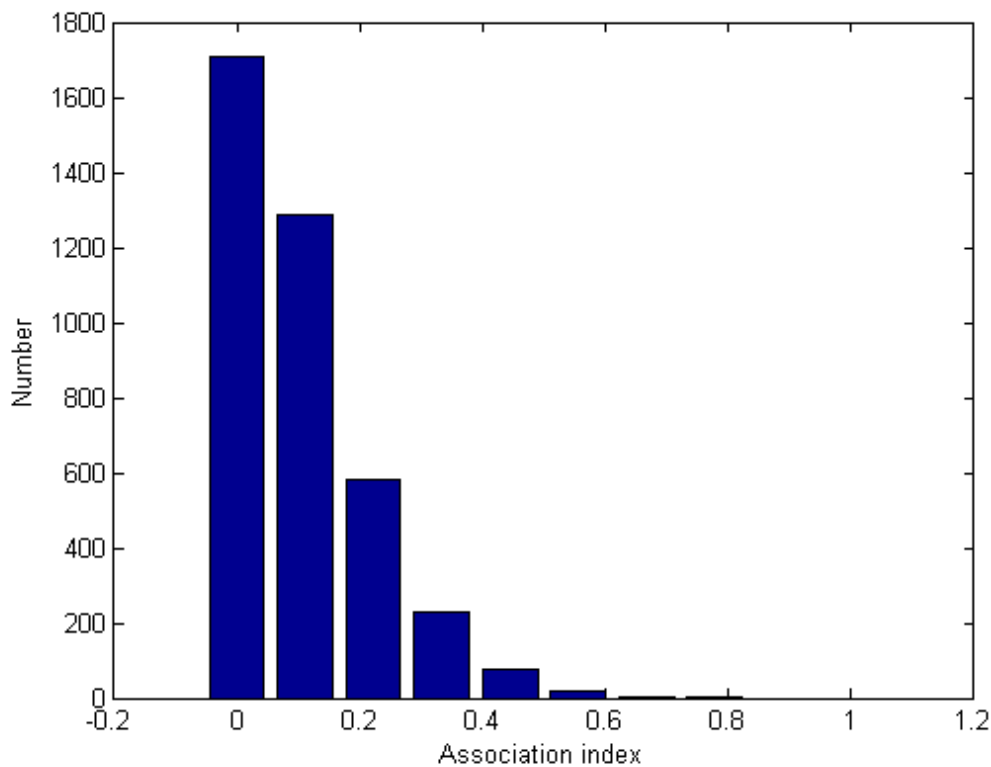


Figure 2. Daily sampling: Distribution of association indices of 63 individuals.

After 20 000 randomizations with a two-sided significance level of 0.05, the expected number of significant dyads was 97.65 but only 24 dyads were significantly different from random. The mean coefficient of association COA or Half Weight Index (HWI) for the East Scottish population was 0.10162.

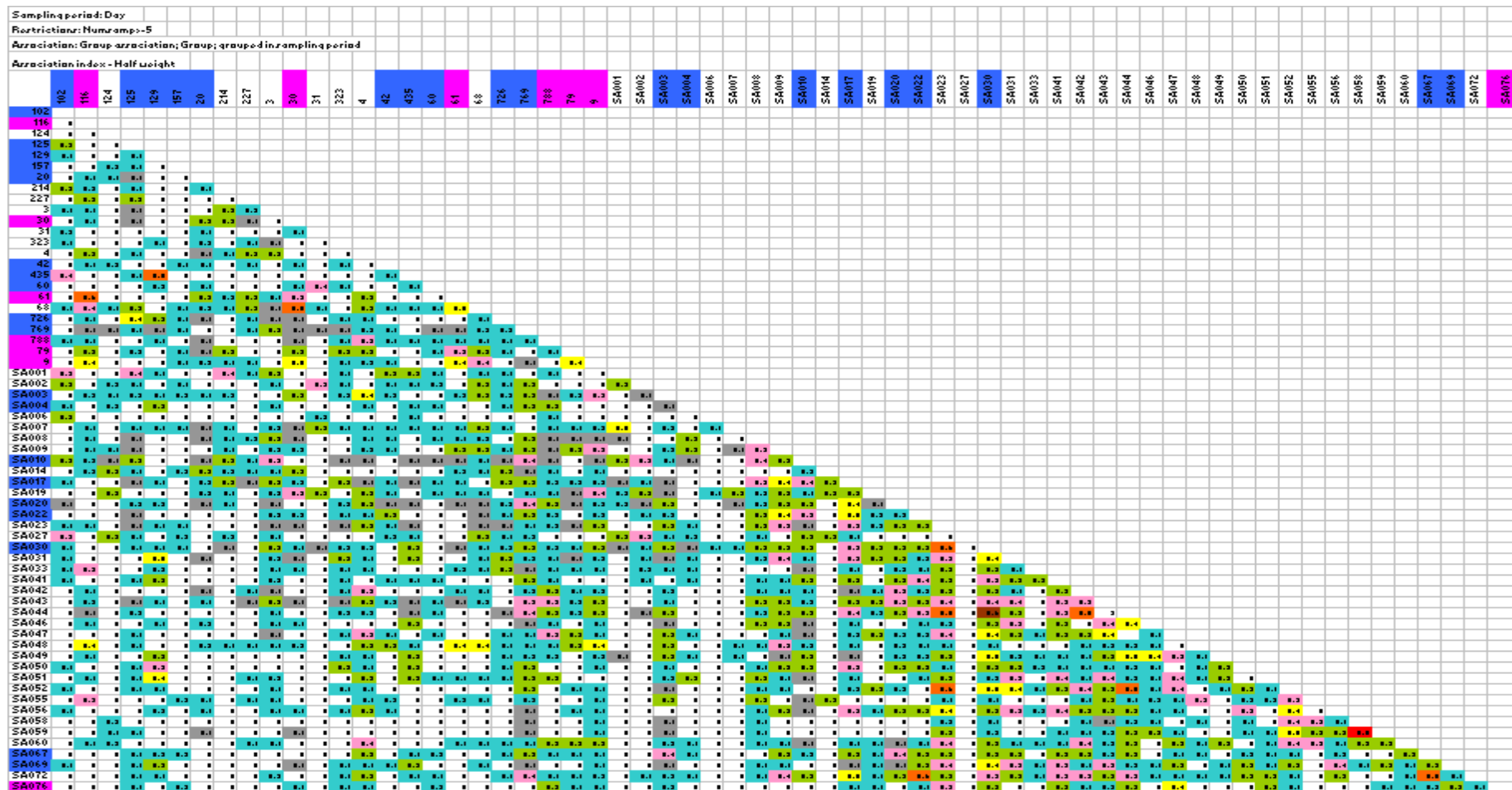


Figure 3. Association coefficients for 63 known individuals with a daily sampling period. Confirmed male individuals are shown in blue and females in pink, both in the top and left hand side of the table. Association coefficients (HWI) = 0.8 are shown in (red), HWI=0.6 (brown), HWI=0.5 (orange), HWI=0.4 (yellow), HWI=0.3 pink, HWI=0.2 (green), HWI=0.1 (light blue) and HWI<0.1 (grey). HWI=0 are left blank.

3.3.3. Biopsying:

Twenty-six successful biopsies were taken in 2006 but two darts were lost in the water. In 2007 twelve successful biopsies were taken and only one dart was lost. A total of 35 samples were obtained from both years. Figure 4 shows the biopsying efforts of the year 2006, it is evident that the efforts were concentrated on the mouth of the river Tay and that dolphins were observed all over the surveyed area.

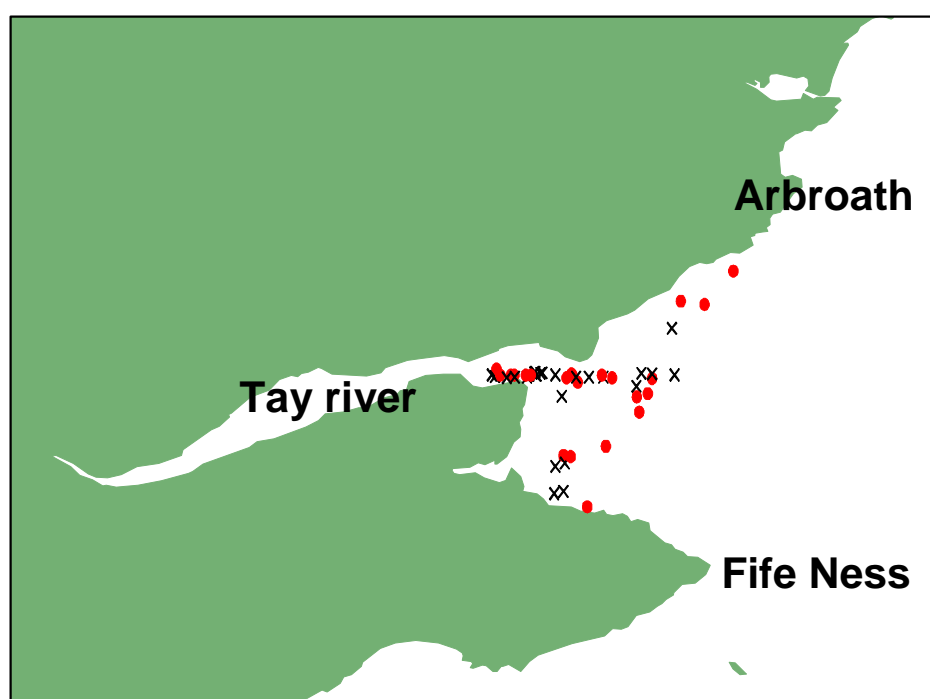


Figure 4. Biopsying efforts of 2006. The red dots symbolize successful biopsies and the crosses unsuccessful attempts. A total of 26 successful biopsies were obtained.

3.3.4. Sexing:

Of the thirty-five biopsy samples obtained twenty-four were confirmed males and eleven females. PCR control reactions were performed with samples of known gender from stranded and captive dolphins from Xcaret Aquatic Park. A female from Wales, a male from the East Coast of Scotland, 2 males and 2 females from Xcaret were amplified in each sexing gel as positive controls

(fig 5). As expected, males showed a double band pattern around 400 bp and females showed only one band. The best resolution of the bands was observed in 2-2.5% agarose gels ran for 40 min at 100 Volts (fig 4).

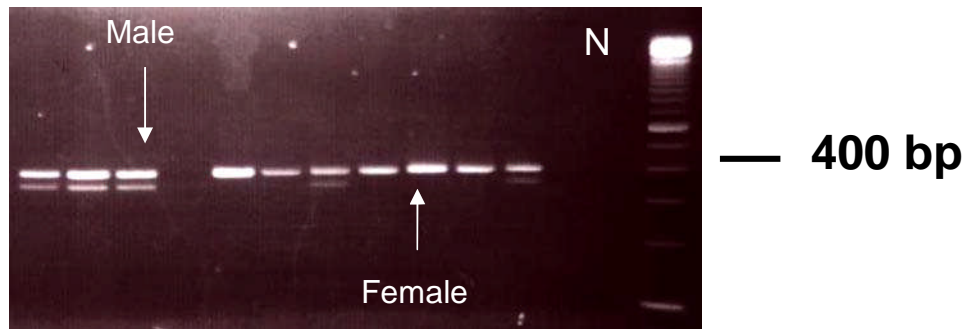


Figure 5. Agarose gel 2% dyed with Ethidium Bromide containing PCR fragments of ZFX/SRY genes of around 400bp. Wells 1-3 contain male positive controls. Wells 5-6 contain female positive controls. Negative control (N) is present beside the size standard ladder. A total of five males and five females are shown.

3.3.5. Relatedness

In order to use microsatellite frequencies to measure relatedness loci should be unlinked, mutations and genotyping errors should be negligible, null alleles need to be absent and markers should not be under selection pressures (Van de Castele et al. 2001). Table 2 shows the Hardy-Weinberg exact test results for all loci in the East Coast population and the error rates calculated for strandings and biopsies. As mentioned in Chapter 2 all loci with null alleles, high error rates and showing linkage disequilibrium were eliminated from this analysis (except Dde70).

Blouin et al. (1996) found that relatedness was measured very accurately in a population of mice with 10 loci and expected heterozygosities around $He=0.74$. To achieve the same results with loci showing $He=0.62$, double the number of loci were required (Blouin et al. 1996). For the East Scottish population

only 5 loci were found with *He* values over 0.6 and only two with *He* larger than 0.75 (Table 2).

Table 2. Genetic diversity and error rate in 13 nuclear microsatellite loci for 63 individuals of the East Scottish population. For each locus: total number of alleles (n), Observed (*Ho*) and expected (*He*) heterozygosity, number of alleles (n) and allelic richness (A). No loci are out of equilibrium in the Table and error rates higher than 20% are shown in bold..

Loci	Number of alleles and allelic richness	Hardy-Weinberg Exact test	Error Biopsies	Error Strandings
D08	(n=3) A=2.2031	He=0.4623 Ho=0.4603 P= 0.6106	0.05	0.1
TV7	(n=4) A=2.005	He=0.4549 Ho=0.4444 P=1.0000	0.05	0.094
TV5	(n=4) A=2.254	He=0.5133 Ho=0.5397 P=0.0295	0	0.222
MK6	(n=6) A= 2.964	He=0.6697 Ho=0.6349 P=0.052	0	0.053
MK8	(n=6) A= 3.216	He=0.7148 Ho=0.6984 P=0.2735	0.088	0.049
MK9	(n=4) A= 1.734	He=0.2840 Ho=0.2698 P=0.0144	0	0.225
Tur117	(n=3) A=1.536	He=0.1888 Ho=0.1746 P=0.0133	0	0.03
Tur48	(n=5) A= 3.072	He=0.6749 Ho=0.8095 P=0.34	0	0.08
Dde61	(n=7) A=2.771	He=0.6478 Ho=0.6667 P=0.1455	0.083	0.035
Dde70	(n=10) A=3.883	He=0.8028 Ho=0.8571 P=0.2304	0.2727	0.155
Tur138	(n=4) A=2.046	He=0.3989 Ho=0.3810 P=0.2056	0.0909	0.019
Tur105	(n=3) A= 2.746	He=0.5420 Ho=0.4762 P=0.5112	0	0
Dde72	(n=7) A= 2.124	He=0.3845 Ho=0.4286 P=0.0104	0.091	0.1

The rarefaction test showed that the calculations of relatedness were very similar with 11, 12 and 13 loci. Changes in the estimation of the relatedness index became stable around 8 loci when a very small standard deviation was found (fig. 6).

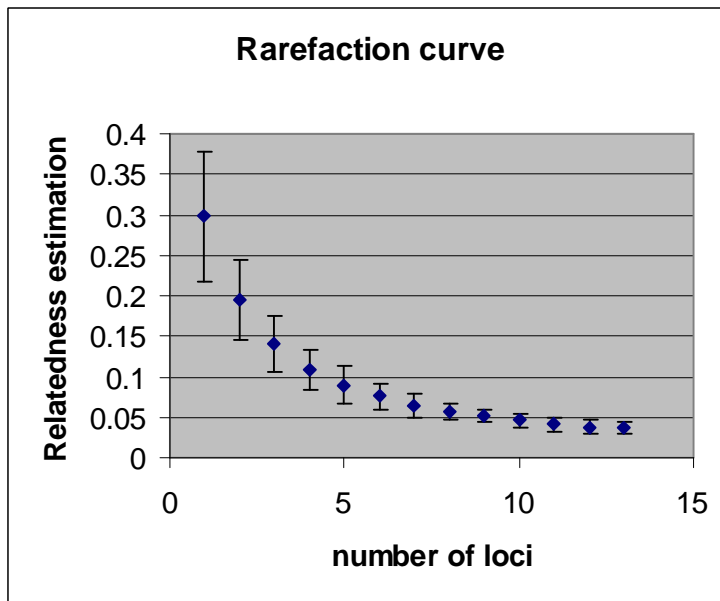


Figure 6. Rarefaction curve. Changes in the estimation of R (relatedness index) and standard deviation.

The normal distribution of these values in the East Scottish population for the Queller and Goodnight index had values ranging from -0.5 to almost 0.9 (Fig 7). The distribution of the values of R for the Lynch and Ritland index did not show a normal distribution and the curve was skewed to the left; values of R ranged from -0.3 to 0.8 (fig. 8).

The value of 0.5 typically represents relatedness values of full-siblings and parent-offspring, 0.25 represents half-siblings, 0 represents unrelated individuals or the background level of relatedness in the population (Queller and Goodnight 1989). Negative values of R indicate individuals that are less related than expected by chance. For the only mother-calf pair obtained in our data set confirmation of allele sharing between them was performed for each of the 13 loci analyzed (Samples M1186/93 (calf) and M0319/98 (mother) were obtained from stranded samples of the Moray Firth). The R value with the QG index was 0.47 while with the LR index was 0.63.

Different arrangements of the East Scottish population were performed. First the East Coast of Scotland was divided in 2 sub-units following Lusseau et al.

(2006). The 'Inner Moray Firth' unit included all the stranded samples that were found in the Moray Firth area, and the 'Outer Community' comprised all the biopsy samples taken in this study plus stranded samples that were found outside from the 'Inner Moray Firth' area.

In general average relatedness seems to be overestimated with the Lynch and Ritland index; female average $R = 0.1859$ (std error = 0.0267) and for males $R = 0.2305$ (std error = 0.0259). While with the Queller and Goodnight index female average $R = 0.0340$ (std error = 0.0159) and for males $R = 0.0347$ (std error 0.0254). The Queller and Goodnight relatedness values between the Inner Moray Firth population and the Outer Community $R = 0.0481$ (std error 0.0276) and $R = 0.0248$ (std error = 0.0284) respectively. Average R values with Lynch and Ritland were $R = 0.1428$ (std error = 0.0274) for the Inner Moray Firth and $R = 0.1519$ (std error = 0.0158) for the Outer Community. The non-normal distribution of the LR index plus the overestimation of R (0.67) for the only mother-calf pair suggests that for our population the Queller and Goodnight index is more suitable than the Lynch and Ritland one.

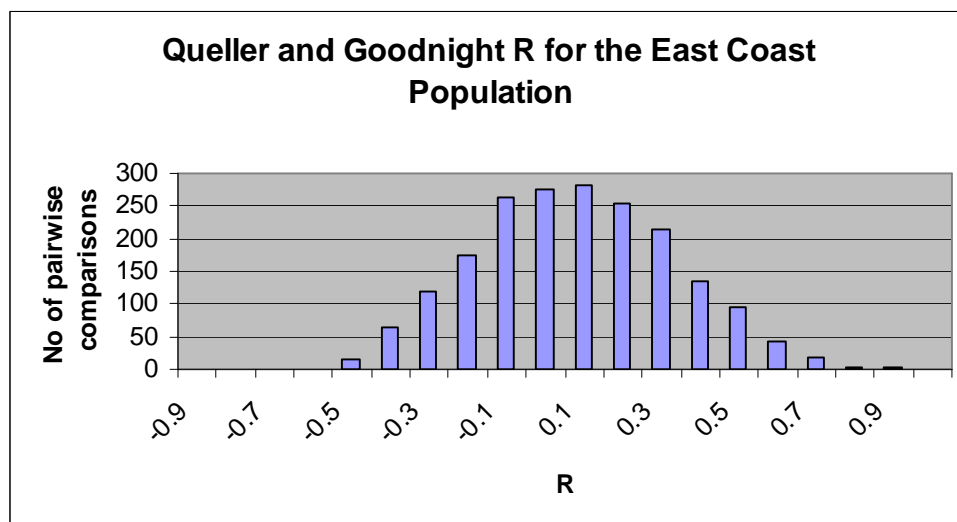


Figure 7. Distribution of the pairwise comparisons for the Queller and Goodnight estimation of R for the 63 individuals of the East Scottish population.

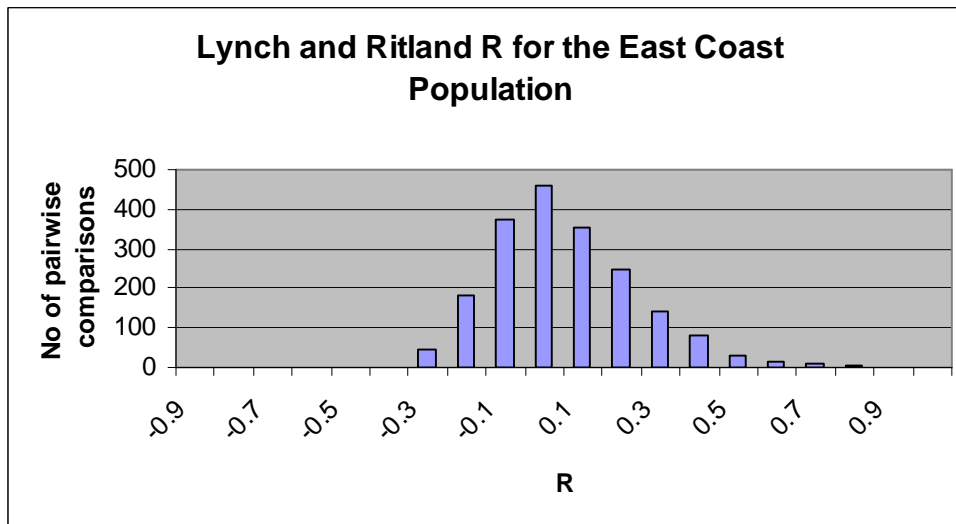


Figure 8. Distribution of the pairwise comparisons for the Lynch and Ritland estimation of R for the 63 individuals of the East Scottish population.

In order to see if there is a correlation between the association patterns of the East Coast of Scotland population and their relatedness a Mantel test was performed. Comparisons were made between a matrix of pairwise associations of the biopsied individuals with the R (Queller and Goodnight 1989) relatedness matrix from the same biopsied individuals. Due to the sampling restrictions (5 days criterion) in the association analysis a total of 27 individuals were compared. There was no evidence for a correlation between association and relatedness in the East Coast of Scotland individuals analyzed. The result of the Mantel test with 10 000 permutations was $r = -0.073$ with a $p\text{-value} = 0.096$.

To standardize the comparison of both indices, all the negative values of relatedness were substituted with zeros, to confirm that the different units were not influencing the negative correlation. The results for the Mantel test with 10 000 permutations was $r = -0.088$ with a $p\text{-value}$ of 0.057. This suggests that there is a slight negative correlation, indicating that more related individuals are less likely to be strongly associated, but the correlation coefficient is too small for this to have a relevant effect.

When comparing the same association matrix with the mtDNA matrix the comparison was reduced to 25 biopsied individuals due to a failure to amplify the mtDNA of two individuals. There was no evidence for a correlation between the mtDNA haplotypes and the association patterns of the individuals from the East Coast of Scotland. The result of the Mantel test was $r = 0.058$ with a *p-value* of 0.205.

Dyads with the highest Half-Weight Indices (HWI) observed for confirmed males and females are shown in tables 3 and 4. Female number 68 was sexed by the presence of calves in the long term photo-ID database of the University of Aberdeen and St Andrews. Values that were found significant after the randomization test (Bejder et al. 1998) are shown with an *. For each dyad the Relatedness Index (R) and the haplotype sharing status (1=shared, 0=not shared) are also shown.

Females with high HWI show a variety of relatedness indices, most of which were similar to halfsiblings (~ 0.25). The only dyad associating significantly (HWI=0.48) after the randomization test (Bejder et al. 1998) showed a relatedness index of almost zero. There is no clear relation with the mtDNA sharing either.

Table 3. Female dyads. Highest Half-Weight Index values and Relatedness Indices (R) for confirmed females are shown. HWI marked with * are values that were significantly different from zero in the randomization test. Shared mtDNA haplotypes = 1, not shared haplotypes = 0

Dyads ID	HWI	R	mtDNA
116/61	0.57	0.1875	1
116/9	0.42	0.3503	1
9/30	0.48*	0.044	0
61/79	0.33	0.2578	1
79/9	0.43	0.1971	1
30/61	0.3	0.2666	0
30/68	0.52	NA	

When looking at male/male dyads showing higher HWI (although not significant) with other males, almost the majority showed negative values of relatedness (R), indicating that the pairs that spend more time together are less related than the average in the population. One pair had a fullsibling index (R=0.464) and another pair showed values of almost halfsibling. There is no clear relation with the mtDNA sharing pattern either. The only male dyad that had a significant HWI, also showed a negative relatedness value. The only 2 significant dyads between males and females that were higher than 0.2 showed also very low relatedness values and their mtDNA could not be compared.

Table 4. Male/male and male/female dyads. Highest Half-Weight Index values, Relatedness Index (R) and mtDNA sharing for confirmed males and females are shown. HWI marked with * are values that were significantly different from zero in the randomization test. Shared mtDNA haplotypes = 1, not shared haplotypes = 0. Individuals 30 and SA076 were females and are shown in bold.

Dyads ID	HWI	R	mtDNA
129/435	0.53	0.1891	1
SA010/769	0.3	-0.0277	0
769/SA020	0.4	-0.1901	1
SA017/SA010	0.4	-0.2121	0
SA017/SA020	0.4	0.0098	1
SA067/SA003	0.3	0.4644	0
SA020/769	0.3*	-0.1901	1
30/20	0.23*	0.0358	NA
SA076/SA030	0.28*	-0.0283	NA

It is important to notice that the sample size of this study was not enough to achieve statistical significance for a positive or negative correlation of association and relatedness. The number of significant dyads after the randomization test was also limited to be able to compare patterns of individual dyads but some patterns can be suggested, such as females forming stronger bonds with other females to which they are related in

different levels. On the other hand males showed also a variety of relatedness levels but mostly lower association and relatedness indices.

3.4. Discussion:

Defining sampling restrictions is crucial for association analyses. Researchers face an obvious trade off between including as many individuals as possible and reflecting the real patterns of association of the population (Chilvers & Corkeron 2002). If individuals that have been seen only a few times are included, the HWI obtained for them will be overestimated. On the other hand, if we restrict the analysis to a large number of times that the individual was recorded, we will lose several associations in the population, depending on the sampling effort.

The cut off for association studies varies from animals that have been seen at least 3 times (Parsons et al. 2003) to animals that have been seen on at least 7 occasions (Moller et al. 2001) or 10 times in a year (Lopez & Shirai 2008; Smolker et al. 1992). Most studies use a cut-off between 4 or 5 days (Lusseau et al. 2006, Santos & Rosso 2008; Chilvers & Corkeron 2002) as was done in this study.

Another important consideration is how many individuals from the population have been identified in each group and included in the analysis. Lusseau et al. (2006) used data from encounters where 50% or more dolphins were identified and were properly marked. Chilvers and Corkeron (2002) reliably identified 57% of the population of bottlenose dolphins off Point Look, Australia and 37% of the groups were fully identified (excluding calves). Santos and Rosso (2008) identified approximately 30% of marine tucuxi dolphins studied in Southeastern Brazil. In my study approximately 48% of the individuals were identified.

Most of the association coefficients (COA) or Half Weight Index (HWI) values in this study were low, between 0 and 0.2. They ranged from 0 to 0.82 but only one dyad of individuals with unknown sex showed such a high value (0.82). Bottlenose dolphins COAs in the Indian River Lagoon (IRL), Florida, ranged from 0.09 to 0.83 too, with the most common values were between for 0.013 to 0.24 (Kent et al. 2008). Despite the fact that the COAs were very similar, the IRL population showed preferential associations within their own sexes, where male-male associations were stronger than female-female associations.

Smolker et al. (1992) also found the most of the dyads in the population ranged between values of 0 and 0.2 and the highest values were mostly for male-male pairs too. In this study, the most of the biopsied individuals are males. High values of associations were not observed between pairs of males. The implications of these results are discussed in Chapter 4. The pairs of confirmed females showed very similar values to the males, suggesting there is no evidence for stronger associations that are sex related.

The mean COA/HWI of my study was 0.10162 and the number of dyads that were significantly different from random was only 24. Overall, individuals in the Eastern Scottish population of bottlenose dolphins do not seem to form strong associations. These fluid patterns of association were also present in other species of dolphins such as tucuxi (*Sotalia guianensis*), in Southeastern Brazil (Santos & Rosso 2008) and in a large community of bottlenose dolphins living in oceanic waters off North Stradbroke Island in eastern Australia (Chilvers & Corkeron 2002). Chilvers and Corkeron (2002) suggest that studies of bottlenose dolphins tend to find stronger associations in small populations inhabiting bays or estuaries; while fluid patterns are present in large populations inhabiting open, deeper waters. Although they point out that these differences might be due to the fact that large, open communities are difficult to sample and the studies might not be reflecting the real

association patterns. This does not necessarily explain the lack of strong associations in the Eastern Scottish population. The population is known to be small, approximately 130 individuals (Wilson 1995; Quick 2006) but ranges over a large area. The average range for an individual is around 123km², which is higher than the ranges observed for other populations of this species (Wilson 1995).

The Queller and Goodnight (1989) relatedness estimator performed better than the Lynch and Ritland (1999) one. Relatedness values for males and females with QG were very similar ($F = 0.0340$ and $M = 0.0347$). The differences between The Inner Moray Firth Community ($R = 0.0481$) seems to be slightly more related than the Outer Community ($R = 0.0248$) but this difference is not very big. The association patterns and genetic relatedness calculated with microsatellites were not correlated according to the Mantel test performed. A lack of correlation was also found between maternal relatedness and association patterns. Details on female dyads showed that animals with higher HWI had a variety of relatedness values. Two of them had values similar to the average of the population $R = 0.1663$, three of them were related approximately like half-siblings or higher and one pair is not related at all. According to Blouin et al. (1996) at least 10 microsatellite loci with $He = 0.75$ are required to accurately estimate relatedness. This was not the case in our study for the East Scottish population which suggests that our estimates of relatedness are most likely underestimated.

In Sarasota Bay females associate in bands constituted by individuals that share calves of the same age (Wells 1986). There is a synchrony in the births of the calves and the females have tight bonds and are philopatric (Wells 1986). This has also been observed in female bottlenose dolphins in Port Stephens, Australia that show higher associations with females that share their reproductive state (Möller & Harcourt 2008). Male-male closest associates also showed a variety of relatedness values, with one pair showing

values typical of full-siblings but most of the dyads showing a complete lack of relatedness.

Mitochondrial DNA analysis revealed that female bands in Sarasota Bay are formed by multiple generations of individuals with different mtDNA haplotypes (Duffield & Wells 2002). In Port Stephens, Australia there was a correlation between genetic relatedness with microsatellites and mtDNA haplotypes between frequent associates but there were also closely related females that were not associated (Moller et al. 2006). The lack of correlation between associations and relatedness was also found in common dolphins (*Delphinus delphis*) from a stranded pod. These findings suggest that other mechanisms, apart from kin, determine the composition of these pods (Viricel et al. 2008).

This is different from other social species that show fission-fusion societies. Wild African elephants (*Loxodonta africana*) who remained in groups with their first order relatives were more likely to fuse with groups that shared the same mtDNA haplotype (Archie et al. 2008). Female rhesus macaques (*Macaca mulatta*) from Cayo Santiago, Puerto Rico (Widdig et al. 2006) and the Himalayan regions of Pakistan and India (Melnick 1987), also tend to form strong associations with their maternal relatives more than with their paternal relatives, but overall with their kin.

This pattern is also present in some delphinid species such as striped dolphins (*Stenella coeruleoalba*). Female striped dolphins form small groups and associate more often with their relatives than males (Gaspari et al. 2007). A similar pattern is present in Atlantic spotted dolphins (*Stenella frontalis*) in the Bahamas. Here higher association values were observed within families than between families (Welsh & Herzog, 2008).

Association patterns were not studied in the population of Barra in Western Scotland, but it has been documented that this population consists of approximately 15 individuals (Grellier & Wilson 2003). All individuals showed the same mtDNA haplotype and from the seven individuals biopsied six were females and one was a male. Relatedness values among these individuals resembled full-siblings values. This evidence suggests that 'Barra' animals show completely the opposite patterns of association and relatedness to the neighbouring population in East Scotland.

This variety of patterns exhibited between different populations and species of cetaceans suggest that other mechanisms apart from kin selection drive association patterns. Tight bonds between preferential associates can be formed due to age class or reproductive state. As female bottlenose dolphins usually have one calf in periods of 2 or 3 years, maternal relatives are not likely to compose age-based groups. On the other hand paternal relatedness cannot be detected by individuals (to the extent of our knowledge), so individuals that have the same father do not know they are related and this is not likely to influence their associations.

When resources are small, dispersed and in patches greater than group size, the shares of the profits just relate to the number of individuals in the group and not to their hierarchy (Hooff & Schaik 1994). Individuals in these groups just have to make sure that the groups are of adequate size and if bigger, that they can exclude other members or move to another group (Hooff & Schaik 1994). If there is no within group competition for resources, there is no need for the development of hierarchies and societies can become non-philopatric (Van Schaik, 1989 in: Hooff and Schaik 1994). Bonds between individuals arise when they live in the same place, when they are philopatric. In mammals dispersal is meant to be carried out mostly by males, while females are supposed to become specialized in foraging and become philopatric (Greenwood 1980, Trivers 1985, Perrin & Mazalov 2000). This is not always

the case in bottlenose dolphin populations. Natoli et al. (2005) found that both sexes in bottlenose dolphins population in the North Atlantic, Mediterranean and Black Sea, show a certain degree of philopatry. They suggested that the structuring of the populations is more related to the environmental conditions that provide their food (Natoli 2005). In these populations we would therefore expect strong and long lasting associations of individuals and the presence of kin selection. The chance for kin selection to act directly in social behaviour is related to the amount of time that individuals in a population spend with their relatives (Maynard-Smith, 1964). If associations are loose in the East Scottish population, related individuals are not likely to be associated together more than it would be expected by chance.

Female alliances are also likely to develop if they need to protect themselves from predators or share resources (Hoof & Schaik 1994). Bottlenose dolphins in the East Coast of Scotland do not seem to have serious predators and they occupy a very wide range with patchy food (Wilson 1995). These two factors could promote the loose nature of female associations in this population and as discussed in Chapter 4, of male associations as well.

Chapter 4 No evidence for male alliances in the bottlenose dolphins of East Scotland

4.1. Introduction:

Promiscuous or polygynous species obtain reproductive success by exploiting different resources; females rely on obtaining food to bring up their offspring, while males compete for a large number of mates (Perrin & Mazalov, 2000). A reproductive strategy that has been documented to aid males obtaining mates is the formation of male alliances. This strategy is present in several vertebrate species such as the lance-tailed manakins, (*Chiroxiphia lanceolata*), where alliances of different levels of duration and strength were displayed by males as a strategy to court females (DuVal 2007). In mammals, male wild lions (*Panthera leo* L.), form groups of 3 or more individuals that compete with other groups of the same size, for the access to female prides. This results in more mates and therefore may produce more surviving offspring (Bygott et al. 1979). Coalitions to gain access to females are also present in male savanna baboons, (*Papio cynocephalus*), where older or middle-low ranking males engage in agonistic behaviour towards younger-higher ranking males (Bercovitch 1988). Male chimpanzees (*Pan troglodytes*) also rely on the formation of alliances as a reproductive strategy (deWaal, 1982 in: Duffy et al. (2007). Alpha males in these alliances obtain the highest mating success but allow their fellow allies to have preferential access to mates (Duffy et al. 2007)

In order to gain access to reproductive females, bottlenose dolphin males establish strong associations with other males, and so form alliances that can last several years (Connor et al. 1992). This behaviour has been well documented in four different populations: Shark Bay, Australia (Connor et al. 1992), Port Stephens, Australia (Möller et al. 2001), Sarasota Bay, Florida

(Wells 1986 cited in: Duffield and Wells 2002) and Little Bahama Bank, Bahamas (Parsons et al. 2003).

In Shark Bay, where bottlenose dolphin alliances were first described, males associate preferentially with other males to herd non-pregnant females. They associate in pairs or in triplets and these associations remain, even when the animals are not herding a female. This kind of association was described as a first-order alliance with association coefficients similar to those of mothers and calves (80-100) (Connor 1992). Association coefficients were calculated following the Half Weight Index (HWI) (Cairns & Schwager 1987). The values of this index go from 0 for individuals that have never been seen together, to 100 for individuals that are always together. These alliances are very stable and can last up to 12 years (Connor 1999). Connor et al. (1992) also found that two first-order alliances, around 5-6 individuals, could join forming a second-order alliance, with the aim of herding a female from another alliance or defending themselves from attacks. The members of a first order alliance were shown to participate in different second order alliances (Connor et al. 1992).

This behaviour becomes more complicated when up to 14 males form a “super-alliance”, to gain access to females. Individuals inside the super-alliance switched partners frequently forming pairs and triplets randomly (Connor et al. 1999).

When the genetic relatedness of males forming alliances in Shark Bay was investigated, individuals constituting the first and second order alliances were proven to be highly related with one another, whilst the members in the super-alliance were no more related than would be expected by chance (Krützen et al. 2003). This evidence suggests that ‘kin selection’ could play an important part in alliance formation, although relatedness is not a condition

for forming alliances in other populations (Duffield & Wells 2002; Moller et al. 2001).

In this context the benefits of an alliance are obvious; they are an important reproductive strategy and they could promote inclusive fitness. This important reason to study alliance formation in other populations of bottlenose dolphins leads us to the question: When should a strong association be considered an alliance?

Connor et al. (1992b) observed that males in Shark Bay associate in groups of 2 or 3 individuals: pairs and triplets. Pairs of males in alliances were each other closest associates; while an individual was included in a triplet if it was the second closest associate of any of the pair members and his HWI was within 20 points from the HWI the pair members had with one another (Connor et al. 1992b). Males in pairs or triplets tend to socialize, forage, travel and most importantly herd females together (Connor et al. 1992b). Möller et al. (2001) followed the same association criteria in a population of *Tursiops aduncus* in Port Stephens to characterize male alliances. The pairs or triplets that were closest associates or second closest associates had to show significantly non-random associations following the permutation test (Bejder et al. 1998) and they should have been observed herding a female (Moller et al. 2001).

The presence of male alliances has also been documented in a bottlenose dolphin population of the Little Bahama Bank in the northern Bahamas (Parsons et al. 2003). This study included animals that had been seen between 1997-2000 at least 3 times, they found 423 groups and identified around 107 individuals. The sex of the individuals was determined by molecular methods, from 21 confirmed males 14 appeared to form only alliances pairs. Males were considered to form an alliance if they followed all the following criteria:

- a) Pairs whose HWI (Half Weight Index) across the 4 years sampling study was greater than the average maximum for all males.
- b) Pairs or trios that associated significantly more than random after the permutation test (Bejder et al. 1998).
- c) Pairs or trios that were reciprocal top associates following Connor et al. (1992b) and Möller et al. (2001).

The presence of alliances has also been reported in Sarasota Bay, where similar high levels of association (HWI between 0.45 and 0.96) and herding behaviour of male pairs was shown to occur between males (Wells et al. 1987). This population has been extensively studied for the last 30 years and long term associations between males have been documented to last up to 20 years (Wells 1986 cited in: Duffield & Wells, 2002).

The ultimate outcome of alliance formation should be reproductive success, and 'kin selection' could be a very plausible explanation for this behaviour. Krützen et al. (2004) showed that some members of first order alliances in Shark Bay had a significantly higher number of offspring, compared to the males that do not form alliances. This pattern would support Hamilton's theory of kin selection, where individuals gain "inclusive fitness" by helping their relatives to reproduce by showing altruistic behaviour (Hamilton 1963). This is especially true in the first order alliances, but it does not explain the formation of super-alliances.

This kin selection pattern is not present in all populations of bottlenose dolphins that form alliances. Möller et al. (2001) found that bottlenose dolphins (*Tursiops aduncus*) in the sandy bay of Port Stephens, southeastern Australia, also show preferential associations with one, two or three other males for herding females. Surprisingly, these alliances are constituted by non-related individuals, suggesting that other mechanisms rather than kin selection is driving the formation of male alliances in this population.

This seems to be the case also in Sarasota Bay where the male alliances were studied using mtDNA and Y chromosome markers. Duffield and Wells (2002) assessed the genetic relatedness between the individuals forming the alliances and found that most of the pairs were not related to each other.

Lusseau et al. (2006) analyzed the social interaction of the Eastern Scottish population in a wider range, including: North Sida, Moray Firth, Spey Bay, Aberdeen and St. Andrews Bay, and suggested that the population is subdivided into 2 sub-units: Inner Moray Firth and Outer Community. These 2 sub-units use the same habitat at different times, suggesting some kind of competition between social groups (Wilson et al. 1997; Lusseau et al. 2006). The relationships in the inner Moray Firth seem to be short-term, although some individuals that were in the same school showed long term associations of around 7-8 years. They showed that the individuals that were usually seen in the inner Moray Firth were not observed in the rest of the areas that have been studied (Lusseau et al. 2006). The two subunits seem to overlap in the summer time but they tend not to interact extensively, these two units are not discrete, rather they overlap by way of a couple of individuals (Lusseau et al. 2006).

At least 65 individuals have been identified that occupy the area around St Andrews Bay. This number is the basis upon which a range of population estimates, of up to 120 different individuals, were calculated (Quick 2006). In this study I show in greater detail the male-male associations of bottlenose dolphins that frequent St Andrews Bay from the period May-September 2003, 2004, 2006 and 2007.

4.2. Methods:

4.2.1. Association analyses

Trips were conducted between Arbroath and Fife Ness when sea state was between Beaufort 0-3 with winds of less than 10 mph and it was dry. Every trip was divided into several 'encounters' throughout the day. An encounter was defined as an individual or groups of individuals that were photographed during a period of time (Bejder et al. 1998 following Slooten et al. 1993). Encounters were up to 60 min long. If a group changed composition more than 50% (50% of its members left), it was considered a new encounter.

Only high quality photographs of well marked individuals were used for the 'Association' analysis. Calves or animals with no obvious marks were excluded. Quality grading of the photographs was completed following Quick's (2006) classification (Appendix A). Only photographs that were marked as grade 3.1, 3.2 or 3.3 of this classification were analyzed.

To obtain a quantitative measure of the level of associations between the individuals of the population, the Half Weight Index (HWI) was calculated with the software SOCPROG 2.3 (Whitehead 1997). To identify significant levels of association between dyads, the permutation test suggested by Bejder et al. (1998) and implemented in SOCPROG 2.3 was applied permuting association values within samples. Tests were two tailed ($\alpha=0.05$) and 5 tests were run with 5000, 10000, 15000 and 20 000 permutations, to see when the p-value of the test stabilized. To avoid autocorrelation bias, sampling was restricted to a daily basis (Smolker et al. 1992; Karczmarsky et al. 2005; Chilvers & Corkeron 2002; Santos & Rosso 2008)(Santos & Rosso 2008) and only individuals that have been seen at least 5 days during the study period were included.

A previous study of the social structure of bottlenose dolphins in the Moray Firth, found no strong or long lasting associations between the individuals of the population (Wilson 1995). In order to investigate if some of these bonds might not have been revealed due to conservative sampling, the test was run twice with different sampling restrictions:

- a) Daily sampling: Individuals that were present in the same group on the same day were associated. Only individuals that were seen more than 5 days during the sampling period were included in the analysis.
- b) Encounter-based sampling: Individuals that were present in the same encounter were associated. This unrestricted option allows for individuals that have been seen in different encounters during one day to be counted every time. Only individuals that were seen more than 5 days during the sampling period were included in the analysis.

Photo-identification and molecular sexing analyses were as described in Chapter 3.

4.3. Results:

Over the four years a total of 61 trips and 182 encounters were recorded. In total 138 well marked individuals were identified and included in the analysis, of which twenty-three were confirmed males. After eliminating individuals that had not been seen more than five days over the four years, association coefficients for 63 individuals were obtained, of which 19 were confirmed males. The distributions of the HWI association indices for both sampling restrictions: 'Daily' and 'Encounter' are shown in fig.1a and 1b.

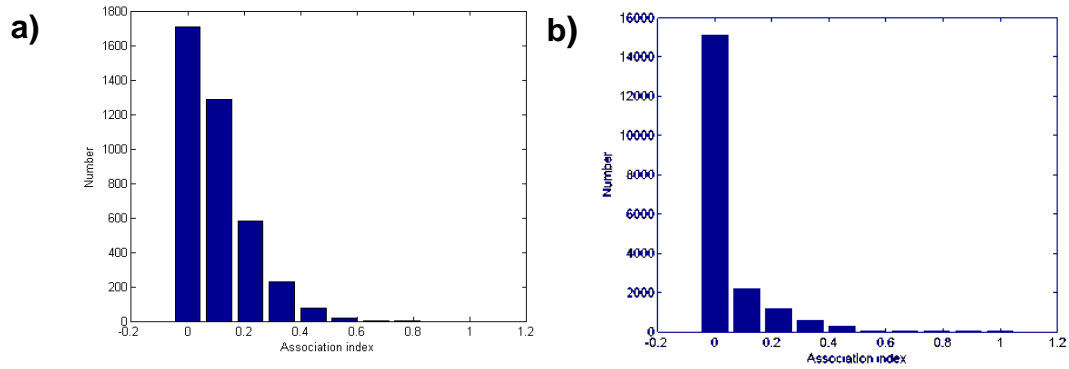


Figure 1a) Daily sampling: Distribution of association indices of 63 individuals. 1b) Encounter based sampling: Distribution of association indices of 138 individuals.

It can be observed that in both histograms the predominant association value is between 0-0.2. The presence of zeros is higher in the unrestricted/encounter analysis with approximate ~15000 records (fig. 1b) vs ~1400 of the restricted/daily analysis (fig. 1a). The highest value in the daily restricted analysis is 0.82 (1a), while in the encounter unrestricted analysis values of 0.8, 0.9 and 1 can be observed (1b).

The details of the association analysis for all twenty-three confirmed males are shown in Table 2. After restricting the analysis to individuals that were seen at least five times throughout the four years analyzed, nineteen males remained Table 2b.

a)

ID	Mean Assoc.	Sum of Assocs	Max. Assoc.
8	0.05	7.72	1.00
102	0.06	9.49	0.43
125	0.06	9.43	0.45
129	0.06	9.11	0.44
137	0.04	5.83	0.44
157	0.02	3.54	0.25
20	0.03	5.71	0.20
42	0.04	5.92	0.29
435	0.05	8.29	0.40
60	0.03	5.12	0.33
726	0.05	8.08	0.45
769	0.07	10.58	0.36
SA003	0.07	10.49	0.32
SA004	0.04	6.01	0.33
SA010	0.07	10.05	0.36
SA012	0.03	4.50	0.44
SA017	0.08	11.58	0.39
SA020	0.08	11.50	0.35
SA022	0.06	8.60	0.48
SA030	0.10	14.33	0.52
SA067	0.07	10.42	0.50
SA069	0.07	10.65	0.40
SA075	0.03	5.70	0.32

b)

ID	Mean Assoc.	Sum of Assocs	Max. Assoc.
102	0.07	5.39	0.35
125	0.09	6.64	0.42
129	0.09	6.73	0.53
157	0.04	3.51	0.18
20	0.06	5.00	0.23
42	0.07	5.29	0.27
435	0.08	5.86	0.53
60	0.06	4.47	0.36
726	0.09	6.56	0.42
769	0.15	10.11	0.39
SA003	0.13	9.34	0.42
SA004	0.06	4.68	0.29
SA010	0.13	9.36	0.39
SA017	0.16	11.06	0.48
SA020	0.14	9.40	0.43
SA022	0.13	8.97	0.57
SA030	0.20	13.59	0.63
SA067	0.12	8.35	0.53
SA069	0.11	7.96	0.44

Table 1. Individuals IDs, mean association coefficient, sum of all associations and maximum association a) Values for twenty-three confirmed males in the unrestricted/encounter analysis, the males in red are the ones that were eliminated in the daily/restricted analysis. 1b) Values for nineteen confirmed males in daily/restricted analysis.

4.3.1 Encounter based analysis

The maximum association value for the individuals that were seen on fewer than five days throughout the whole study were (HWI= 1.0) between individual 8 and an individual of unknown sex (49), this was the highest value obtained but the individual was present in the study only four days. For individual 137 and SA012 the highest coefficient was HWI= 0.44 and for SA075 is HWI= 0.32 with a presumed female 4. The highest values for pairs

of confirmed males in this analysis were: 8 with 102 (HWI= 0.43), 8 with 125 (HWI= 0.38), 137 with SA012 (HWI= 0.33) and SA076 with SA020 (HWI= 0.17). After the permutation test suggested by Bejder et al. (1998) and implemented in SOCPROG 2.3 there were no significant dyads after 20 000 permutations in the unrestricted data set. All the individuals that were seen less than five days during the sampling period were eliminated from the analysis to allow for comparisons of particular dyads.

4.3.2. Daily basis sampling

HWI values for the 63 individuals analyzed are shown in a colour coded pattern in fig. 2 and fig. 3. The confirmed males are shown in blue in the top and left hand side of the matrices. Most of the association coefficients of a confirmed male are between 0.1 and 0.2 as is the case for most of the population shown also in the histograms (fig.1a and 1b).

The highest association coefficient found between 2 individuals of unknown sex was 0.82 (daily sampling) and 0.87 (encounter sampling). If these individuals resulted to be males, this would be the strongest case of an alliance in the East Scottish population of bottlenose dolphins. None of the dyads were significantly different from random in the preferred/avoided companionship test for the unrestricted analysis based on 'Encounters'.

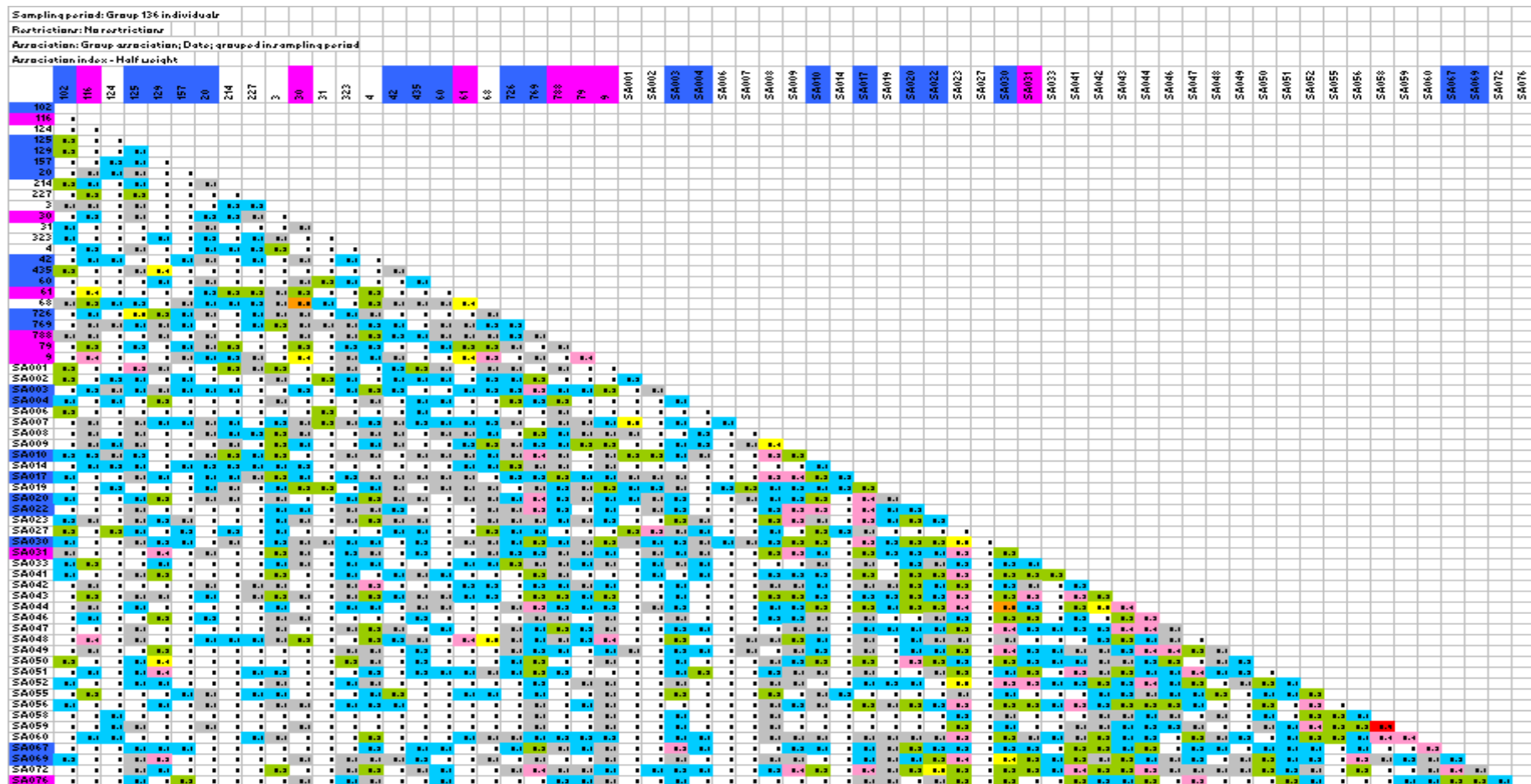


Figure 2. Association coefficients for 63 known individuals with an encounter-based sampling. Confirmed male individuals are shown in blue and females in pink, both in the top and left hand side of the table. Association coefficients (HWI) = 0.8 are shown in (red), HWI=0.5 (orange), HWI=0.4 (yellow), HWI=0.3 pink, HWI=0.2 (green), HWI=0.1 (light blue) and HWI<0.1 (grey). HWI=0 are left blank.

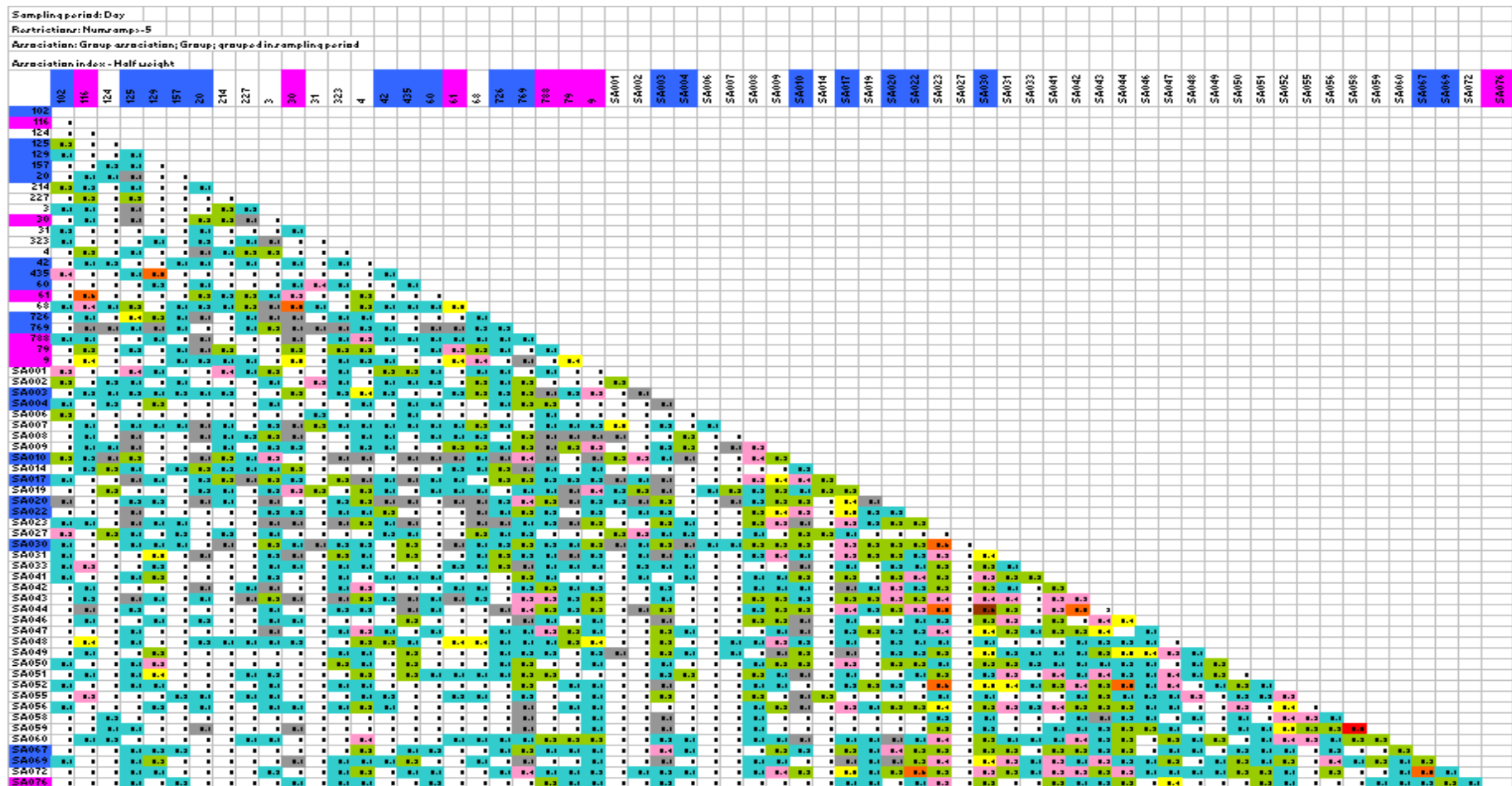


Figure 3 . Association coefficients for 63 known individuals with a daily sampling period. Confirmed male individuals are shown in blue and females in pink, both in the top and left hand side of the table. Association coefficients (HWI) = 0.8 are shown in (red), HWI=0.6 (brown), HWI=0.5 (orange), HWI=0.4 (yellow), HWI=0.3 pink, HWI=0.2 (green), HWI=0.1 (light blue) and HWI<0.1 (grey). HWI=0 are left blank.

In the restricted 'daily' set, the mean for all the associations was 0.10162 and all the values for the 63 individuals analyzed are shown in fig. 3. We can observe that the highest value for a male is 0.63 (brown) and that few values are above 0.5 (red).

The permutation test was carried out 5 times with 5000, 10000, 15000 and 20000 permutations. Each test was repeated 10 times. The *p-values* for all the trials are shown in Table 2. We can observe that at 15 000 and 20 000 permutations the *p-value* stabilizes.

Number of permutations	p-value mean	p-value SD	p-value CV
5000	0.57060	0.98740	0.98720
10 000	0.48150	0.99630	0.99660
15 000	0.56147	0.99853	0.99853
20 000	0.56275	0.99965	0.99975

Table 2. Summary of permutation tests for the Preferred/Avoided companionship test. *p-values* for the comparison of real and randomly originated data. For each set of permutations the test was repeated 10 times. Large *p-values* indicate large real value compared to random values.

After the permutation test suggested by Bejder et al. (1998) and implemented in SOCPROG 2.3, only 24 dyads were found significant after 20 000 permutations. The highest HWI for a dyad that was significant was SA059 and SA058 (HWI=0.82), unfortunately both individuals are of unknown sex. For dyads where at least one animal was a confirmed male the highest value of a significant dyad was between SA030 and SA023 (HWI=0.56), unfortunately SA023 is also of unknown sex. Significant dyads with HWI< 0.50 between a confirmed male and an unknown individual or a female were the following: 30 and 20 (HWI=0.23), 323 and 20 (HWI=0.18), SA003 and 4 (HWI=0.42), SA052 and 435 (HWI=0.00), SA033 and 726 (HWI=0.27), SA072 and SA017 (HWI=0.45), SA049 and SA030 (HWI=0.47), SA076 and

SA030 (HWI=0.28). Individuals 30 and SA076 are confirmed females by means of PCR and 4 is a presumed female, consistently seen with a calf throughout the study.

The significant dyads of pairs where both animals were confirmed to be males were: SA004 and 102 (HWI=0.13), SA003 and 125 (HWI=0.16) and SA020 and 769 (HWI=0.38).

4.4. Discussion:

These results show that while both analyses portray the general pattern of the associations of the individuals in the same way, most of the values obtained are between 0-0.2 in both cases. The unrestricted analysis is more likely to obtain high coefficient values (fig 1.). However this could be a product of coincidental sighting of two individuals only in a few days of sampling, or even of the lack of a good quality photograph of both individuals in different groups. For this reason it is recommended that the sampling restrictions include animals that have been seen at least 5 times during the study period as well as a daily sampling period instead of an encounter based one (Chilvers & Corkeron 2002; Karczmarski et al. 2005; Lusseau et al. 2006; Santos & Rosso 2008).

It is clear that the HWI values for the confirmed males or in general for all the individuals in the population are not close to the values observed for male alliances in other populations. In Shark Bay the values range from HWI=0.80-1.0 (Connor 1992) and in the Bahamas the association coefficients of the male-male pairs ranged between HWI=0.53-1.00 (Parsons et al. 2003). Association coefficients up to 0.96 between adult males were reported by Wells et al. (1987) in Sarasota Bay.

In my study I found only one pair of individuals with a HWI=0.82. This was the highest association value of both individuals. Unfortunately the sex of both is unknown. For the confirmed males, the highest value of a significant dyad was HWI=0.56 between SA030 and SA023. Unfortunately the sex of the latter is also unknown. The rest of the significant values after 20 000 permutations for males paired with either males or females, were lower than 0.5.

Wilson (1995) proposed that the lack of male alliances in the Moray Firth could be explained by the depth of the water where the animals live. Shark Bay waters are shallow which allows males to be able to restrict the movement of females to facilitate copulation. On the other hand the deep waters in East Scotland would not allow this manoeuvre. Second, the male: female ratio of the East Scottish population is unknown, but it is possible that there are enough receptive females in the population for the males to avoid confrontation. Lastly, predation by sharks is an important threat in Shark Bay, whilst the Moray Firth population seems to be lacking predators.

A very important feature of the male alliances is the behavioural component. Males in alliances are often seen 'herding' females (Connor et al. 2000a; Connor et al. 1992a; Connor et al. 1992b; Connor et al. 2000b; Moller et al. 2001; Parsons et al. 2003). In our case we are not including behavioural data in these analyses so the latter cannot be taken into account at this stage, but it could help elucidate the nature of the stronger bonds shown by males in the East Scottish population.

Lusseau et al. (2006) found that most of the associations in the Outer Community (which includes St. Andrews Bay) were short (just a couple of days) and the longer term ones were up to five years. It is difficult to know precisely the age of the individuals in this study which showed the highest association coefficient (0.82). It is likely that they were not among the oldest

males of the population. All of them were identified in St Andrews for the first time in 2003. A possible scenario could include younger individuals forming stronger associations over shorter periods of time (4 years). This way they could try to compete with older/bigger males of the population for the access to reproductive females.

In Doubtful Sound, Lusseau (2007) showed that males with stronger associates (higher association coefficients), were less likely to suffer from aggression from other males. This could be another explanation for the cases of high association coefficients in the Eastern Scottish population. Younger individuals might use this strategy to protect themselves from aggressive behaviour from bigger males. If these strong associations are opportunistic and occur only during brief periods of time, the association analysis employed might not be able to detect them. It would be interesting to compare the association levels of younger male dyads with the ones from older males on a yearly basis. Aggressive displays were often seen during the encounters and aggressive behaviour towards other species and infanticide has also been documented for this population (Patterson et al. 1998).

In Sarasota Bay male alliances are formed between sub-adult and adult males. Long lasting associations (up to 20 years) are of individuals of the same age group (adults or sub-adults) (Wells 1986), but not between adult-sub-adult (Scott et al. 1990). An age class division of the male alliances has also been contemplated as an explanation for the lack of kin selection in male alliances in *Tursiops aduncus* in southeastern, Australia (Möller et al. 2001). Male alliances in this population were not between kin, despite the fact that there were relatives between alliances, bigger dominant males might not want to be in alliances with a younger brother that is two or three years younger (Moller et al. 2001).

The lack of male alliances in the East Scottish population could be related to the lack of strong bonds that the whole population show (except mother-calf pairs) (Chapter 3). Bonding arises when there is philopatry (Hooff & Schaik 1994) and variation in philopatry is related to the type of competition for the resources (Van Schaik 1986 in: Hoof & Schaik 1994). In Shark Bay molecular analysis of mtDNA and microsatellites indicated that dispersal by females seem to be more restricted than in males (Kruetzen et al. 2004). The development of bonds between males in this population is influenced by female philopatry which results in alliances that are kin related (Kruetzen et al. 2003).

If there is no within group competition for resources, there is no need for the development of hierarchies and societies can become non-philopatric (Van Schaik, 1989 in: Hooff & Schaik 1994). The confirmation of gene flow between neighboring populations in UK waters was not possible, but there is some evidence that individuals visit the West Coast of Scotland (Robinson et al. 2009). A female stranding from the English Channel has the dominant haplotype of the East Coast of Scotland and a high degree of relatedness with East Scotland (Chapter 2). This could be evidence of East Scottish individuals migrating throughout UK waters.

Another possible explanation for the lack of male alliances in this population could be related to population structure and competition. Neighbouring populations that are highly structured consist of individuals that potentially compete with each other for resources (food and mates). These 'foreign' individuals pose a threat to paternity within populations. The Little Bahama Bank show strong population structure between the two subpopulations that inhabit the area caused by limited gene flow between neighbouring populations (Parsons et al. 2003). This strong structure might promote the formation of alliances to restrict the access to reproductive females of males of the other sub-population. The Sarasota Bay population consists of around

100 individuals and it seems to be relatively closed (Wells et al. 1987). There are records of Sarasota males associating with individuals from other communities (Wells 1986). Males from these other communities represent competitors for access to females, therefore promoting the formation of alliances. The need to restrict the access to reproductive females from males of a 'foreign' population is not present if a population is isolated, like in the case of the East Scottish population (Nichols et al. 2007; Parsons et al. 2002) and the Doubtful Sound population (Lusseau 2007). In Shark Bay an isolation by distance scenario is present; significant F_{st} values were found mostly between non neighbouring localities and dispersal by females seem to be more restricted (Kruetzen et al. 2004).

Maternal relatedness seems to be very important in the formation of alliances. In Little Bahama Bank, male alliances were significantly correlated with relatedness levels calculated with both mtDNA and microsatellites. All male alliances had the same mtDNA haplotype and this was proportionally higher than in the rest of the population (Parsons et al. 2003). In Sarasota Bay there seems to be a multigenerational female kinship, which was discovered due to an extra chromosome in the population (Duffield & Wells 2002). The males that form the alliances in Sarasota bay are not closely related, but they are part of matrilineal lines that have been close for several generations (Duffield & Wells 2002). There is a synchrony in the births of the calves, the females have tight bonds and are philopatric. Thus, the males have known each other since they are calves, and are more likely to form affiliative bonds that could result in alliances (Wells 1986). Male first order and second order alliances in Shark Bay are also kin related (Kruetzen et al. 2003).

As mentioned above, male bonding seems to be promoted by male philopatry (Hooff & Schaik 1994). Bottlenose dolphins seem to be able to recognize kin, as they remain close to their mothers when their siblings are born (Wells 1991). Signature whistles of male bottlenose dolphins calves share features

with their mother's signature whistle (Sayigh et al. 1995). Because signature whistles aid individual recognition (Janik 2000; Janik et al. 2006; Janik & Slater 1998) it is quite likely that they will recognize their maternal kin and could be able to form kin related bonds (Möller et al. 2001).

Tursiops aduncus males in Port Stephens Australia do not form alliances with either their maternal kin or any other relative, despite the presence of relatives in the population, sometimes in other alliances. Females in Port Stephens are highly associated, they form bands of both related and unrelated individuals, so calves are likely to form stable bonds from either (Möller et al. 2001b). Parsons et al. (2003) suggested that the lack of relatedness between members of male alliances in Port Stephens, is due to the fact that there is little genetic variance in this population. Among twenty sampled males they found only three 3 mtDNA haplotypes (Möller, et al. 2001) while in Little Bahama Bank six haplotypes were found in 21 males (Parsons et al. 2003). If all the individuals are highly related, there is no direct benefit in forming kin alliances, as they all might be closely related. 'Altruism directed at one close relative should not occur at the cost of an equally close relative' (Keller 1997). On the other hand if maternal relatedness explains alliance membership, individuals in antagonistic alliances could be paternal brothers (Connor 2001).

This idea could help explain the complete lack of alliances in very small and isolated populations like Doubtful Sound and the Moray Firth. The Doubtful sound population is composed of 65 individuals and it does not interact with other populations (Williams et al. 1993). Even though a high level of genetic diversity was found in Fjordland (Doubtful Sound and Jackson Bay) (Tezanos-Pinto et al. 2009), the fact that the population is so small and closed could cause the same effect as the lack of genetic variance, not promoting altruistic relationships like male alliances. The East Scottish population is composed of approximately 130 individuals (Wilson 1995 and Quick 2006). Several genetic studies have found a very low level of genetic diversity both

in mtDNA (Parsons et al. 2002, Chapter 2 in this study) and microsatellites (Nichols et al. 2007; Natoli et al. 2003, 2005 and Chapter 2 of this study) suggesting that the population might be inbred, isolated and locally adapted (Nichols et al. 2007) which could explain the lack of altruistic relationships such as male alliances under those principles.

In Chapter 2 it was suggested that male individuals from the Eastern Scottish population might be driving genetic flow between Eastern Scotland, Western Scotland and Wales. If this is the case, males might not need to develop strategies such as male alliances to compete for other females as they will invest more energy in moving between populations and their reproductive success will be given by their ability to interact with different populations (Perrin & Mazalov 2000).

The reproductive success of males and females from several species of mammals is based on their different needs. Females mostly focus on obtaining food to maintain their offspring and males need to focus on mating (Perrin & Mazalov 2000; Trivers 1985). While food can be divided between several individuals, fertilization cannot be shared and this conflict results in male coalitions (Hooff & Schaik 1994). If food patches are dispersed over a large area consequently females will be dispersed. Males then would have to disperse to have access for females.

Lastly there are no records of bottlenose dolphins in the East Scottish Coast before the late 1800s. On the other hand the Flixborough population might have gone extinct at least 100 years ago. This extinct population shares the most common haplotypes with the extant East Scottish population nowadays (Nichols et al. 2007). We can picture a scenario where a small group of individuals from Flixborough started colonizing these waters around 100 years ago. These few individuals adapted successfully to the stressing environment of the East Coast and the population started growing. In the

beginning those few individuals needed to concentrate on survival and reproduction and competition might have not been intense. In 100 years only around 10 generations of bottlenose dolphins would have inhabited these waters. It is possible that the social system of this population is developing into a more complex one with strong and long lasting bonds. Although there is no current evidence for male alliances in this population, if the population continues growing in isolation from neighbouring populations, the access for females could become restricted and lead to the formation of male alliances as a reproductive strategy.

4.4. Concluding Remarks:

Association patterns can give insights into the social structure of a population. Individuals have benefits from living in groups and associating with each other. These benefits are mostly aligned with foraging, protection and reproduction. Association patterns in the East Scottish population of bottlenose dolphins are not strong; they constitute a fluid society with males and females possibly associating in a similar way.

Most of the long term bottlenose dolphin populations that have been studied, like those in Sarasota Bay, Florida, USA (Scott & Wells 1990) and Shark Bay, Australia (Smolker 1992; Connor 2000), have revealed the presence of stronger bonds between pairs or trios of males. Forming 'alliances' is thought to help in gaining access to females and obtaining mates in a very competitive environment, but it has also been hypothesized that alliance partners can convey protection from predators. Wilson (1995) suggested a lack of strong associations in the Moray Firth, but could only speculate on the gender of individuals through behaviour or the presence of calves. A direct observation of the genital slit was only possible in some cases. He suggested that there were several possible reasons for such differences between the Moray Firth and the Shark Bay populations.

First, the depth of the water in Shark Bay allows males to be able to restrict the movement of females to facilitate copulation, while the deeper waters in East Scotland would not allow this manoeuvre. Second, in Shark Bay there are different levels of alliances that compete for access to females (Connor et al. 2001; Connor et al. 1999; Connor et al. 1992b). The male: female ratio of the East Scottish population is unknown, but it is possible that there are enough receptive females in the population for the males to avoid confrontation. Lastly, predation by sharks is an important threat in Shark Bay, whilst the Moray Firth population seems to lack predators.

There are several novel contributions of my work to this field of study. My study comprises the biggest sample set obtained to date for bottlenose dolphin populations around the UK including both stranded and biopsied individuals. This was the first time that animals in Scotland have been sexed with molecular techniques.

Population structure analyses were not conclusive establishing a strong connection between the East Coast of Scotland, Wales and part of the West Coast of Scotland. Relatedness analyses on the other hand showed the possibility of a small proportion of East Coast migrants into Wales. Males from the East Coast of Scotland could be driving gene flow into Wales and the West Coast. They could be investing more energy in dispersing instead of competing among themselves for access to females; this strategy could also help the population to avoid inbreeding depression. It is clear that they do not need to defend their territory, as I found no indication of migrants from other populations.

From my study, I can add two more possible explanations for the lack of alliances in the East Scottish population of bottlenose dolphins. Firstly, Keller (1997) suggested that altruistic behaviour does not represent a gain if the cost is obtained from one close relative against another. The lack of genetic diversity and the small size of the population suggest that all the individuals of this population are highly related. Relatedness analyses show that the average relatedness value is similar to the ones shared by half-siblings. The presence of only a few loci with H_e values higher than 0.75 suggest that our estimates are most likely underestimated, which means that the average relatedness of the population is even higher. High relatedness values mean that there would be opposing relatives in antagonistic behaviours; this could explain the absence of alliances.

I found even higher values of overall relatedness in the Welsh population. Thus, it would be very interesting to study the association patterns of that population. I would predict that they also lack altruistic behaviour and strong associations.

The second possible explanation for a lack of alliances relates to the ranging patterns of individuals as revealed by genetic analysis. Previous studies of the population structure of bottlenose dolphins around UK waters have suggested that the East Scottish population is isolated and locally adapted (Nichols et al. 2007). Concerns about its viability are obvious as being small and isolated; the chances of extinction are increased. Parsons et al. (2002) suggested a connection between the East Coast of Scotland and the Welsh population that could not be confirmed due to her small sample size. Further studies also suffered from sample size issues that they tried to solve by pooling samples in a putative population called 'Outer UK', composed of the West Coast of Scotland, Wales, English Channel and Ireland. Pooling all these populations together Nichols et al. (2007) did not find a strong connection between them and the East Coast of Scotland. I found a small connection of Welsh individuals with the East Coast of Scotland, therefore confirming Parsons et al. (2002) finding and suggesting rather the presence of small gene-flow or an ancestral connection. The presence of one stranding sample in the English Channel that is highly related to individuals in the East Coast of Scotland population, suggests that females could also be travelling long distances. Bottlenose dolphins in this population show larger ranges than other populations, this is meant to be due to the patchy nature of the resources (Wilson 1995). These patterns of food dispersal drive the individuals to maintain groups that are adequate for foraging independently of their hierarchy, therefore promoting the presence of relaxed association patterns, they do not need to worry who they associate with, but it is important that the number is adequate for obtaining their food.

One of the most striking findings of this study is the autonomy and composition of the West Scotland population of Barra. Previous population genetic studies found a very high genetic diversity that was assumed to be related to the occasional influx of pelagic individuals. Those samples came from stranded animals of unknown origin. Strandings can come from geographically distant areas, carried to shore by oceanic currents. Grellier and Wilson (2003) reported a small size (approx. 15 individuals) for this West Coast population, and its constant presence throughout 3 years. Biopsies from 7 individuals suggested that the population is composed of highly related females. Many new questions remain: Is this group of individuals a real population or only a sub group? How does this population survive and how long has it been occupying the Sound of Barra?

The complex dynamics found off the West Coast of Scotland resemble those of the Western North Atlantic, where coastal and pelagic individuals exist in sympatry (Hoelzel et al. 1998). All the scenarios with the Bayesian clustering analysis as well as the relatedness tree, suggest that the West Coast of Scotland is divided into 2 groups and that most of the individuals from Barra belong to a cluster that has a connection with Wales. The other half of the population belongs to a cluster that has a connection to the English Channel. Microsatellite analyses suggest that this cluster is connected to the pelagic populations off Portugal and Spain (Natoli et al. 2004).

The very different origins of the East Coast of Scotland/Wales cluster and the West Coast of Scotland cluster are clear when we look at the haplotype network. The most likely explanation is the occurrence of two different founder events in the past and a more ancestral status for the East Coast of Scotland cluster, this is also suggested by the higher scores of (φ_{st}) obtained compared with (F_{st}). In the present these divergent populations seem to be in contact. It is crucial to define the present migration rates between these populations. Unfortunately the sample sizes from the West Coast of Scotland,

Wales and England are still too small to obtain good estimates for migration rates.

Nichols et al. (2007) suggested that the populations inhabiting the UK waters constitute a meta-population with complex dynamics of extinction and re-colonization. They found that an extinct population of Flixborough originated from the same matri-lineage as the East Scotland population. The autonomy of the Flixborough population was revealed when the population structure was analyzed with microsatellites in a Bayesian clustering framework. It is unknown why Flixborough went extinct but the extreme range that this and other UK clusters inhabit and their very small sizes raise concerns about their viability.

On the other hand, if the Flixborough population went extinct 100 years ago; this is around the same time that bottlenose dolphins in the East Coast of Scotland appear in the records of naturalists. With this basis, we can picture a scenario where few individuals of Flixborough survived by moving to the East Scottish population. This few individuals became very successful in colonizing these waters due to the presence of abundant sources of food, no predators and no conspecific competitors. The patchiness of the food resources influenced the presence of relaxed association patterns and the small numbers probably reflected on their lack of dispersal. If present individuals of the East Coast of Scotland are travelling to the West Coast of Scotland and beyond, it is not likely that molecular markers can pick up this geneflow in only around 10 generations.

5.1. Future work

Almost 30 years of photo-identification efforts have been carried out on the East Scotland population of bottlenose dolphins. This is one of the best studied populations of bottlenose dolphins along with the ones in Sarasota and Shark Bay. These efforts should be used to investigate detailed changes in the association patterns of the population.

For example, association patterns of the same individuals could be compared between the Moray Firth and St. Andrews. Most importantly changes in association strength could be studied for pairs of related individuals, to look at the development of strong bonds. It could also be interesting to look for possible temporal 'alliances' in younger individuals to compete with the biggest males of the population. Furthermore, studying the reproductive success of males in the population could help to elucidate the lack of male alliances.

With a bigger dataset the amount of individuals that will be eliminated from the Association analysis after a 5 days restriction sampling period will decrease and a more reliable determination of the social structure of the population will be acquired. A more even distribution of biopsies between males and females could also aid defining the gender biased associations and dispersal. The lack of biopsied mother-calf pairs stopped me from determining closer relationships among individuals.

It would be interesting to know which males visit Wales and Barra, if this is a regular activity. Do migrating males form alliances in other populations to gain access to females or do they remain solitary? Do migratory males have a better reproductive success than the resident males of those populations? The sex determination with genetic markers, allows several hypotheses to be confirmed about males being heavily marked or bigger in size. It also allows

the gender determination of juvenile animals that do not show strong scars or presence of calves. It is now possible to determine the type of interactions young individuals have with their own and opposite sex. With this information it is possible to investigate the early stages of social bonds and their development.

It is important to calculate the amount of current gene flow between these populations inhabiting the extreme range of the distribution of the species with a bigger sample size from Wales and the West Coast. If the East Scottish population has inhabited these waters for only 100 years; this gives us an invaluable opportunity to get an insight into the development of bottlenose dolphin social systems, their consequences in the evolution of populations and its genetic outcome.

Appendix A: Data Management

St Andrews area Encounter Sheet

Date: _____ Start time: _____
Shot information: _____
Daily Encounter No: _____ Depth (m): _____
Lat/long: N _____ Sea State: _____
W _____ Wpt no. _____
No of individuals: MIN _____ MAX _____ BEST _____ Complete Y/N

Notes:

If reencounter of an already biopsied animal:

ID: _____ Shot information _____

Comments:

SURFACING	DIVES	GROUP	MOVEMENT	BOWRIDING
Slow	long	bunched	progress	play/fight
Medium	short	subgroups	same spot	tailslap
Rushing	altern	dispersed	fish	jumps

End time: _____ End shot: _____

Location: N _____ W _____

Wpt no. _____ Depth (m): _____

Photographer: _____ Crew: _____

Encounter No. _____

Trip No.: _____

St. Andrews area Genetic Biopsying.

Date: _____ Name of recorder: _____

Sea State: _____

Name of biopsy taker: _____

Shot information: _____

Dart #: _____ Dolphin ID: _____

Time of the shot: _____

Successful/ Unsuccessful _____ Sample #: _____

Gun settings: _____

Distance to the dolphin: _____

Angle of impact: _____

Clockwise position towards the boat:



Photographs or Video:

Behaviour within 5 minutes after the biopsy:

- I. No visible reaction
Dolphin continued prebiopsy behaviour

II. "startle" response
but stayed in the immediate vicinity of
the boat

III. startle and mild acceleration

IV. startle and fast swimming
(white water or porpoising)

Single leap
Multiple leap
Tailslap
Change dir

<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>
<input type="checkbox"/>

Group composition as in encounter Y/N?

Comments:

Encounter No.

Trip No.

Quality grading system
Modified from Quick, 2005.

Grade 1

*Picture not in focus

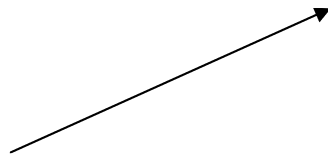
*Fin height smaller than 1 cm



Grade 2

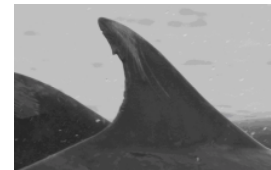
*Fin is perpendicular

*Entire fin is in frame

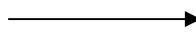


Grade 3

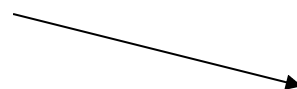
*Dull light (Grade 3.1)



*Backlit/silhouette (Grade 3.2)

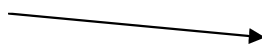


*Bright light clear image
(Grade 3.3)



Grade 4

*Image not perfect but you can
identify individual



Appendix B: Behavioural responses to biopsying and wound healing rates.

Report on bottlenose dolphin reactions to remote biopsy sampling

Vincent M. Janik and Valentina Islas
Sea Mammal Research Unit, University of St Andrews

PPL # 60/3135

In 2006 we took 26 skin biopsies from 26 different bottlenose dolphins along the Scottish coast between Fife Ness and Arbroath. This was licensed under the Home Office project licence number 60/3135. The delivery of this report is a requirement stated on this project license. It summarizes the observable effects of our efforts on the dolphins.

Behaviour

As part of our sampling we monitored the behaviour of our chosen target animal in the 5 min period after the sample was taken. Since we had to identify animals before we took a sample, all animals were travelling in parallel to the boat in the minutes before the sample was taken.

We divided the post-biopsy behaviour in 4 main categories: no visible reaction, startle, startle combined with a mild acceleration, and startle combined with fast swimming. Additionally we recorded the presence of single or multiple leaps, tailslaps or the complete change of direction of an animal (fig. 1).

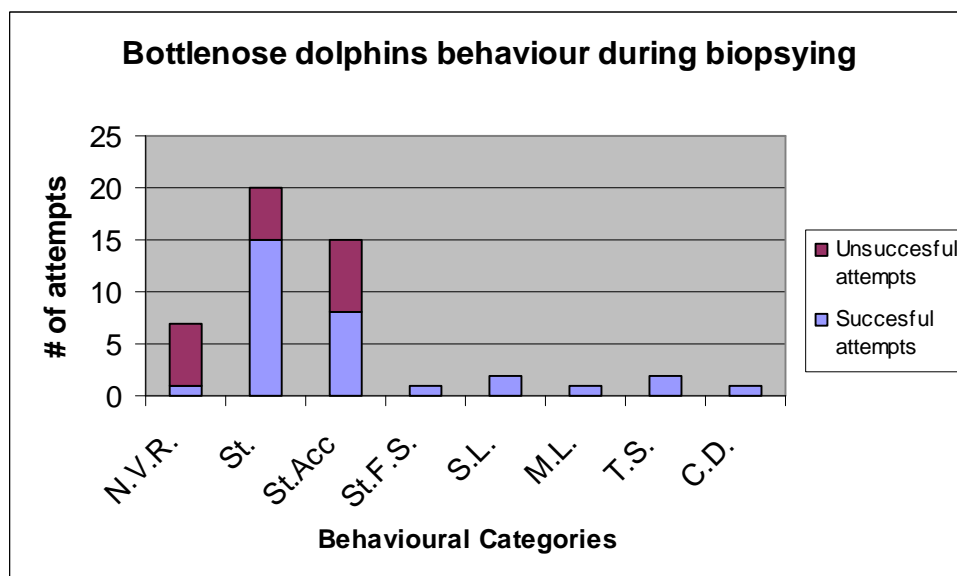


Figure 1. Categories of dolphin behaviour during biopsy. N.V.R. (no visible reaction); St. (startle); St.Acc (startle and acceleration); St.F.S. (startle and fast swimming); S.L. (single leap), M.L. (multiple leap); T.S. (tail slap) and C.D. (change direction).

As shown in fig. 1 most of the responses to the procedure were “startle” and “startle with acceleration”, even when the sample attempt was unsuccessful. This suggests that the startle response is primarily a reaction to the acoustic component of the procedure. One individual did not react at all to the biopsy. For six animals we could only observe the immediate reaction (which was a startle and mild acceleration) since we lost track of them before the 5 min post-biopsy period was over. Three of these animals were seen to join larger groups. All observed dolphins returned to their previous swimming speed within the 5 min period after the biopsy.

Some stronger reactions (leaps, tail slap, etc) were also observed. We should point out that leaping is common in these dolphins and that we were unable to confirm the identity of leaping animals. Thus, the percentage of the leaps observed after a biopsy was taken that were actually carried out by the biopsied animal is unknown, since all biopsied animals were part of larger dolphin groups. We were able to take a post-biopsy photograph of 12 individuals during the five minutes of post-biopsy observations.

Post-biopsy behaviour in odontocetes is generally reported to be “mild”. A common response is a startle or flinch, which was also observed in our study. A 19% of the individuals showed none visible reaction to the procedure. The main response of killer whales (*Orcinus orca*) after a biopsy consisted in “shake and acceleration” immediately after the shot but they would return to their normal behaviour by their next breath (Barret-Lennard et al. 1996). This was considered a “slight” response and it was present a 74% of the times. Just a 6% of the individuals reacted in a “strong” way, consisting in a continuous shaking under the water and for subsequent surfacings and 1% showed “other behaviour” being evasive prior to biopsy and reacting very strongly to a “miss” (BarrettLennard et al. 1996).

Weller et al. (1997) also biopsied bottlenose dolphins (*Tursiops truncatus*), they found reaction 100% of the times for a short-term. The authors divided the possible behaviour in four categories: 1) no reaction, 2) low-level reaction (dolphin changed its behaviour in a mild way), 3) Moderate Reaction (changed behavior in an observable but short-term manner) and 4) Strong reaction (behaviour dramatically modified). All the individuals showed a moderate reaction startle response, even when 50% of the hits actually had a tissue sample, 25% of the hits had no sample and the other 25% of the hits consisted of the bolt striking the water prior to the animal, resulting in no sample retrieval (Weller et al. 1997).

Other techniques of tissue collection for genetic analysis of free-ranging dolphins have proven to be useful. Bilgmann et al. (2007) biopsied common and bottlenose dolphins, while they were bow-riding, with a pole system. The system retrieved a 5 mm diameter and 1 cm long sample. Response categories were divided as follow: 0) no noticeable reaction 1) flinch, but individual continues bowriding, 2) individual accelerates under water and leaves the bow, 3) individual accelerates, leaves the bow and leaps and/or porpoises, 4) individual accelerates, leaves the bow and shows multiple leaps and/or porpoises. In total of the 4 populations analyzed (2 of bottlenose

dolphins *Tursiops spp.* and 2 of common dolphins *Delphinus spp.*) the main reaction was an acceleration under water departing from the bow (2). This reaction was present 64% of the times while obtaining a biopsy and 12% without obtaining a biopsy. A 17% of the time they showed “no reaction” and no strong response was observed.

A less invasive procedure has been tested in dusky dolphins (*Lagenorhynchus obscurus*) (Harlin et al. 1999). Skin swabbing in bow-riding dolphins showed a successful collection of tissue in most cases (78%) that was suitable just for amplification and sequencing of mitochondrial DNA. The behavioural responses were divided in: Move left or Move right (dolphin moved from position at the bow after contact), Dive (dolphin dove directly under the bow), Startle (dolphin flinched in response to contact), flight (dolphin fled from the boat in a prolonged surface-active swimming behaviour), tailslap (dolphin flexed its caudal region and brought it forcefully down), Increase speed (dolphin swam faster for a short term) and No response (dolphin did not changed behaviour during contact sampling) (Harlin et al. 1999). An 11% of the contacts resulted in “no response”, 89% responded in a “mild” way moving to the left or to the right of the bow. Stronger responses like tailslap and startle occurred just once in 114 contacts (Harlin et al. 1999).

Parsons et al. (2003b) compared the effectiveness of invasive (biopsy) and non-invasive (collection of feces) sampling techniques for molecular analyses in bottlenose dolphins from the Bahamas. During the survey period they collected 25 biopsies and 44 fecal samples, 66% of the fecal samples could be assigned to individuals and the DNA yield was just enough for mtDNA amplification. Costs of fecal laboratory analysis were four times more expensive than the ones for biopsy techniques. The behavioural responses to the biopsy sampling were divided as follow: no visible reaction=15%, slight=34% (flinch and/or immediate dive), minor=34% (tail flick/kick and immediate dive), moderate=6% (tail slap and acceleration away from vessel), strong=9% (breach) and persistent 0% (reaction to biopsy vessel persists

beyond immediate encounter). Slight and minor reactions were shown approximately in the same frequency for successful and unsuccessful attempts.

The most relevant comparison for our results is with a study by Krützen et al. (2002) since they used the same PAXARMS system on the same species of dolphin. They found that the dolphins' main response to biopsying was "mild", involving their first two reaction categories: startle, remaining in the vicinity of the boat (58.5%) and splashing with occasional tailslaps with or without coming back to the boat (31.6%). This is comparable to what we have found in our study. Since we did not stop the boat after the biopsy was taken, we cannot compare boat approaches.

Strong responses in Krützen et al. (2002) consisted of "single leaps" and were shown in 2.1% of biopsies. The strongest response was multiple leaping found in response to 3.8% of the biopsies.

(d) Healing rates

During the survey period we re-sighted twelve individuals after the day they were biopsied, six of these individuals have a good photograph post-biopsy of the wound and the other six were just identified to be present in an encounter. We got good photographs of the biopsy wounds for 12 individuals, nine of them taken on the same day and the rest 6, 11 (fig. 2c) and 25 (fig. 2e) days after the biopsy was taken. Wounds varied in their shape and size, depending on the impact in the animal (fig. 2).

From the 26 biopsies taken, just three of them consisted of a "big" sized tissue (fig. 3); twelve were "good" biopsies (fig. 2a, d and f); five were small (fig. 2b and 2c) and four were scratches (fig. 2e). The wounds pattern we observe for specially the big sized biopsies, is consistent to the one observed by Weller et

al. (1997) “ oval shaped wound, deeply pink to red in colour, several mm deep, no other apparent discoloration”.

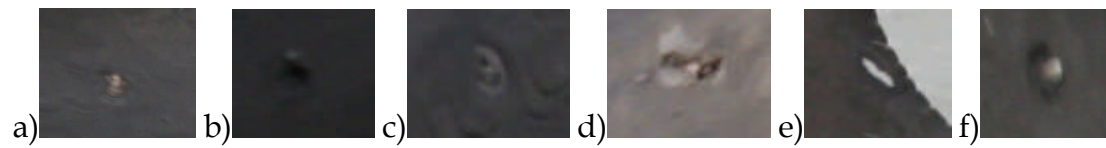


Figure 2. Different types of biopsy wounds. a)MF3, 7days after being biopsied, b)MF102 the day of the biopsy c)MF129, 11 days after biopsy d)MF726 the day of the biopsy e)MF769, 25 days after biopsy f)SA010 the day of the biopsy.

Particularly one animal was seen very often (MF435) and it gave us the opportunity to follow the development of its healing. We can observe in fig. 3 the wound immediately after the biopsy was taken on the 27/07/06.



Figure 3. MF435 just after been biopsied on the 27 07 06

The next day after its biopsy (28/07/06), we encountered MF435 again and the area of the biopsy seemed to be depressed but no sign of infection or swollen was observed (fig. 4). In a similar case where a “large” sample was taken, Krutzen et al. (2002) have the following description: “Initially we could observe only a sickle-shaped black mark that probably originated from the edge of the flange, and a dark spot in the centre where the sample had been taken”.

Weller et al. (1997) description for the same period is “an oval shaped wound, pinkish to white in colour, darker spot at centre of wound, skin at edge of wound”.



Figure 4. MF435 on the 28 07 06. A slight depression can be observed in the area of the biopsy wound.

After 21 days on the 16 of August 2006, we encountered MF 435 and as we can see in (fig 5.) the wound was all covered in epidermis with the centre of the wound still red. The same was found by Krutzen et al. (2002) after 18 days. For a period of 15-26 days, Weller et al. (1997) described the wound as follow: “pinkness absent, oval shaped wound, white in colour, darker spot at centre of wound surrounded by lighter gray halo”.



Figure 5. MF 435 on the 16 08 06. White epidermis along the edge is observed.

We encountered MF435 for the last time on the 25 08 06, thirty days after its biopsy. The animal came close enough to the boat to be photographed several times from its left hand side. Unfortunately the right hand side of the animal, where the wound was located was exposed to a bad light, and the behaviour of the group didn't allow us to remain in that side for a long time. The group consisted of several mothers with calves so we didn't want to harass them. Even when the photograph shown in (fig. 6) is not very good, we can appreciate how the wound is all covered in white and no signs of infection can be detected. Krutzen et al. (2002) found that after 25 days the wound was completely covered by new epidermis while Weller et al. (1997) said that between 40-42 days post biopsy, there was just a "white spot, no discoloration or epidermal depression". It is important to observe that the system employed by Weller caused a much bigger wound (3 to 4 cm deep).



Figure 6. MF435 30 days after biopsy.

So far the healing rates and the behaviour post-biopsy are very similar to the ones found by Krutzen et al. (2002) and Weller et al. (1997) as well as the fact that there was no sign of infection in any of the wounds observed. Krutzen et al. (1997) were able to follow 4 animals every day and they found that after 23 days the wound was covered in new epidermis and that it started re-pigmentation after 36 days, while for Weller et al. (1997) it was after 61 days that the wound was nearly normally pigmented.

We can expect that as in previous studies where biopsy sampling is employed the population will just have a short-term reaction as it has been shown so far in our research. No signs of infection or change in the behaviour were noticed.

Report on bottlenose dolphin reactions to remote biopsy sampling in 2007

Vincent Janik and Valentina Islas

During the year 2007 we did 20 trips along the coastline from Fife Ness to Arbroath from which we took 12 skin biopsies from 12 different individuals previously identified. During 2006, forty-eight attempts (both successful and unsuccessful) were carried out, while in 2007 we did just twenty-four. The particularly bad weather conditions of this year resulted in a bigger sampling effort and a smaller sample size, as well as a poor record of the biopsied animals. The weather conditions made very difficult the follow of the individuals as well as the attempts to try to photograph the wounds.

a) Behaviour

The summary of reactions of the 20 biopsy attempts we did during 2007 is shown in Fig.1. Following the same protocol of the year 2006, we divided the behaviours in four categories: no visible reaction, startle, startle with acceleration, startle and mild acceleration and startle with fast swimming. We also recorded: multiple leaps, tailslaps and change of direction of the animals.

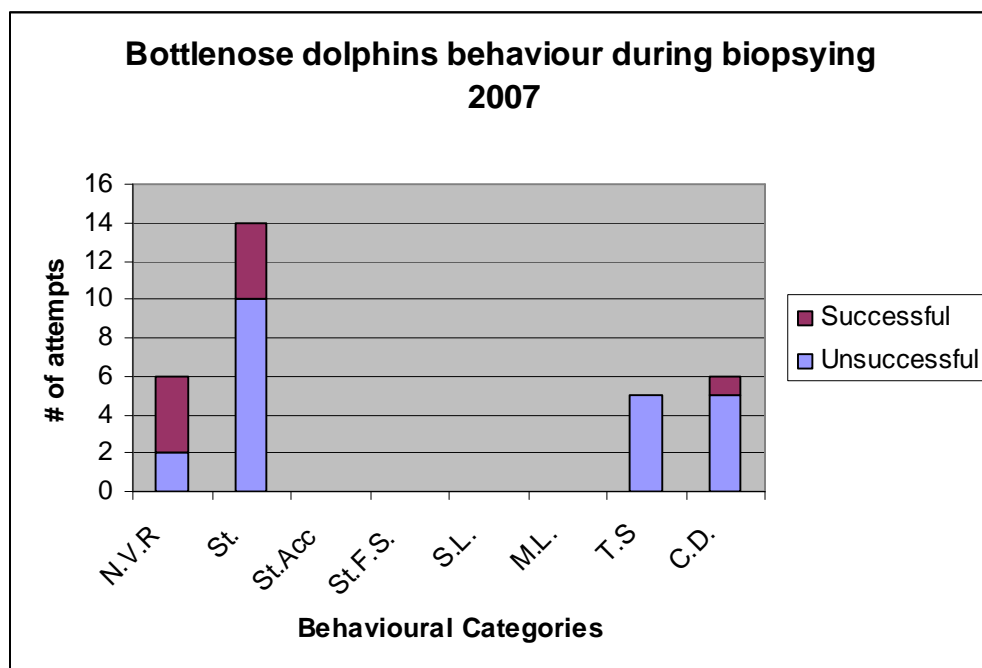


Figure 1. Categories of dolphin behaviour during biopsy. N.V.R. (no visible reaction); St. (startle); St.Acc (startle and acceleration); St.F.S. (startle and fast swimming); S.L. (single leap), M.L. (multiple leap); T.S. (tail slap) and C.D. (change direction).

The most of the reactions to the biopsy, both successful and unsuccessful, resulted in a “startle” reaction, as it is shown in Fig.1. This percentage is consistent with the behaviour observed last year (2006) for the same procedure, which again suggests that the reaction of the individuals could be also due to the acoustic component of the biopsy. We also found that in five occasions the “startle” reaction was followed by “tailslaps”. (Krützen et al. 2002) showed that bottlenose dolphin main response to the biopsying procedure was “mild”, involving their first two categories: “startle, remaining in the vicinity of the boat” (58.5%) and “splashing with occasional tailslap with or without coming back to the boat” (31.6%), similar to our results. Also consistent with the percentages observed in 2006 is the percentage of “no visible reaction” mainly to unsuccessful attempts.

The main differences in the percentages of behaviours observed from the biopsied animals between 2006 and 2007 are the increase in tailslaps and change of direction of the animal. One reason for the differences in stronger

reactions, particularly “tailslaps”, observed between both years, could be related to the lack of experience of the members of the crew in 2007, this can also be related to the complete absence of “startle” reactions combined with mild or fast acceleration, possibly mixing these two behaviours occasionally. It is also noticeable that during 2007 any of the biopsied individuals, or animals forming part of their group, showed any kind of leaps.

b)Healing rates

To continue with the protocol of 2006 we tried to photograph the wounds of all the biopsied individuals from both years 2006 and 2007. Eighteen of the twenty-four individuals sampled in the year 2006 were re-sighted in the study area in 2007. Fig.2 shows photographs from 2007, of the biopsy wounds of animals sampled in 2006.



Figure 2. Biopsy wounds of individuals biopsied in 2006, the scars are pointed with a red circle. The photographs were taken on the 08/08/2007 and 17/07/2007 respectively from left to right.

As we can see in the two individuals of Fig. 2, after a year the wounds developed into a noticeable scar with no signs of infection and the animals are seen in the vicinity of the boat. Although the size of the scars is noticeable, it is important to point out that the low temperature of the water, where these animals live, can be constraining the regeneration paths of the epidermal cells

Feltz and Fay 1996 in Wilson et al. (1999) leaving big scars, even when the damage to the skin from the biopsy was not that deep.

Of the twelve individuals biopsied in 2007, just three were observed the same day they were biopsied. Attempts to photograph the fresh wound were successful just in two cases (Fig.3). In both cases we can observe a small black dot, with no swelling or depression of the area surrounding it.

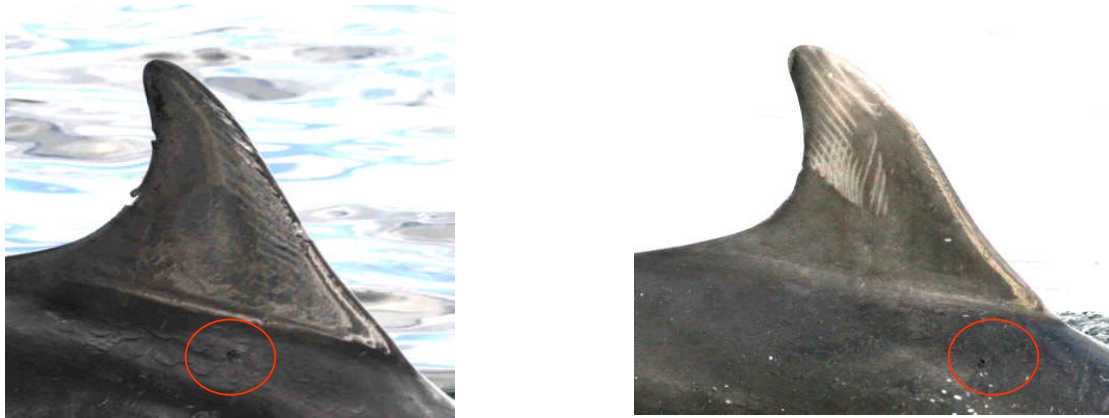


Figure 3. Biopsy wounds of individuals biopsied in 2007, the scars are pointed with a red circle. These wounds correspond to the day the animals were biopsied.

Follow-ups of the healing rates of the individuals biopsied in 2007 were extremely difficult mainly due to the weather conditions. In several occasions there were several weeks between each trip and the probability of the animals changing their location was very high. Five individuals were photographed in trips after they were biopsied. One of them was re-sighted, three days after it was biopsied, other three were re-sighted after nine days (Fig. 4), fourteen days (Fig.5) and twenty days post biopsy. The last one was re-sighted again 46 days after the biopsy was taken (Fig. 6).

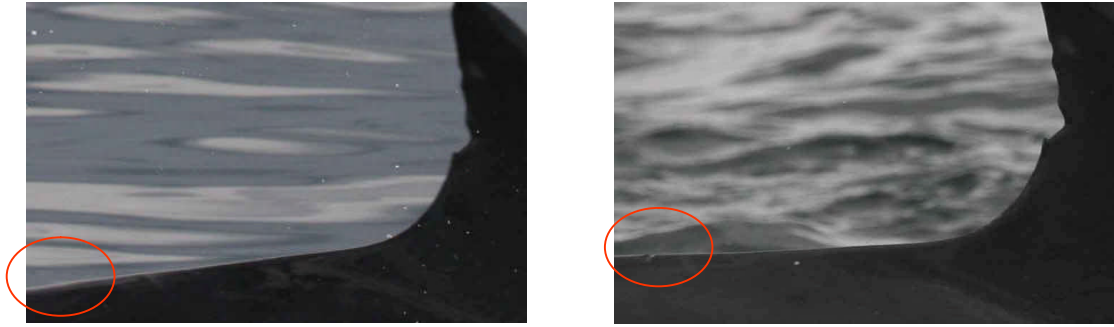


Figure 4. On the left we observe the skin of the individual before the biopsy, and on the right we can observe the small depression that corresponds to the biopsy wound nine days after the individual was biopsied.



Figure 5. Biopsy wound after fourteen days.

In Fig. 4 we can see that there is no swelling or infection around the area of the wound. In Fig 5 we can observe the developing of the white tissue consistent with the regeneration at this state of the healing process (Krützen et al. 2002). Fig. 6 shows the biopsy wound of an individual that was biopsied on the 23/07/07, seconds after the biopsy was taken and the wound was fresh. Better pictures of the biopsy wound were not taken, as we lost the animal immediately after the biopsy. This individual was observed again 46 days later, on the 08/08/07. We can observe that the wound is covered in white new epidermis and no signs of infection can be detected, consistent with what (Krützen et al. 2002) found after 25 days in Shark Bay, Australia.



Fig.6. Photograph taken 46 days post-biopsy on the 08/08/07 of a biopsy wound, we can observe the obvious white circle of new epidermis, covering the wound.

Appendix C: Microsatellites

Table 1. Microsatellites origins and PCR details. The original reference, repeated motif, primers sequences, annealing temperature and reported sizes along with number of alleles (n) are shown for the 20 microsatellites used in this study.

Name and author	motif	Sequence 5' 3'	Tm °C	Product size (n)
KWM12a (Hoelzel et al. 1998a)		F-CCATA-CAATCCAGCAGTC R-CACTGCAGAATGATGACC	46	~ 250 bp (7)
TexVet 5 (Rooney et al. 1999)	(CA) ₂₄	F-GATTGTGCAAATGGAGACA R-TTGAGATGACTCCTGTGGG	51	236-260bp (9)
TexVet 7 (Rooney et al. 1999)	(CA) ₁₂	F-GCACTGTAGGGTGTTCAGCAG R-CTTAATTGGGGCGATTTCAC	54.5	155-163bp (6)
D08 (Shinohara et al. 1997)	(TG) ₁₈	F-GATCCATCATATTGTCAAGTT R-TCCTGGGTGATGAGTCTTC	56	~130bp (8)
D22 (Shinohara et al. 1997)	(CA) ₃ -TA- (CA) ₂₁	F-GGAAATGCTCTGAGAAGGTC R-CCAGAGCACCTATGTGGAC	57	~135bp (7)
MK6 (Krützen et al. 2001)	(GT) ₁₇	F-GTCCTCTTTCCAGGTGTAGCC R-GCCCACTAAGTATGTTGCAGC		145-189
MK8 (Krützen et al. 2001)	(CA) ₂₃	F-TCCTGGAGCATCTTATAGTGGC R-CTCTTTGACATGCCCTCACC	56	87-119bp (11)
MK9 (Krützen et al. 2001)	(CA) ₁₇	F-CATAACAAAGTGGGATGACTCC R-TTATCCTGTTGGCTGCAGTG		168-180
EV37 (Valsecchi and Amos, 1996)	(AC) ₂₄	F-AGCTTGATTTGGAAGTCATGA R-TAGTAGAGCCGTGATAAAGTGC	57	~250bp (8)
EV1 (Valsecchi & Amos 1996)	(AC) ₁₃ (TC) ₈	F-CCCTGCTCCCCATTCTC R-ATAAACTCTAATACACTTCCTCCAAC		115-197
Tur4_80 (Nater et al. 2009)	(GATA) ₁₀	F-AGCCAATGTCAGGGTGCTGGAT R-GGGGCTTCTTGGCCTCTGTAA	60	287-335
Tur4_91 (Nater et al., 2009)	(GATA) ₁₄	F-GTTGGCTCTCCAGCTCTCAGGT R-CAGTGGCTCCCATCTGTATTAGTCA	60	207-235
Tur4_117 (Nater et al. 2009)	(GATA) ₉	F-TTGCAGTCAGCGTTTTCCAGAGA R-GCCAGCCCATCCTTCAGATTTC	60	175-187
Tur4_138 (Nater et al. 2009)	(GATA) ₉	F-GTGGCTTACCATGGTGGATTTCAG R-GCATGGCCATAAAGGGAGGAG	60	207-227
Tur4_105 (Nater et al. 2009)	(GATA) ₁₁	F-CCCCGGCCTGCTTACCTCTG R-CCGCCCCCTCCCCAAGTC	56	367-403
Dde59 (Coughlan et al. 2006)	(GATA) _n	F-TACACAGCTTACTTACCTTACCAA R-GTCCCTTTGAGCAGAGTTCTA	56	384-432
Dde61 (Coughlan et al. 2006)	(CTAT) ₈	F-CTGAACCTGAGTTCGGTAACA R-TGAGCAATACACATATGCACCT	55	128-172
Dde70 (Coughlan et al. 2006)	(CA) ₂₁	F-ACACCAGCACCTACATTCACA R-TCAGCAGCATTCTAACCAAC	56	133-161
Dde84 (Coughlan et al. 2006)	(CA) ₂₂	F-AATAATCCTTTGTGGTTTCTGTT R-CATTCCAGGTACAGCTTTTCA	56	148-166

Microsatellites genotyping

Raw data was obtained from the Automatic sequencer Beckman Coulter. An example of the output is seen in Fig. 1. The size of the allele is usually determined by the highest peak read by the Beckman for each dye in the range reported for the particular microsatellite. I only allowed peaks with 'peak height' higher than a 1000 to be scored as alleles.

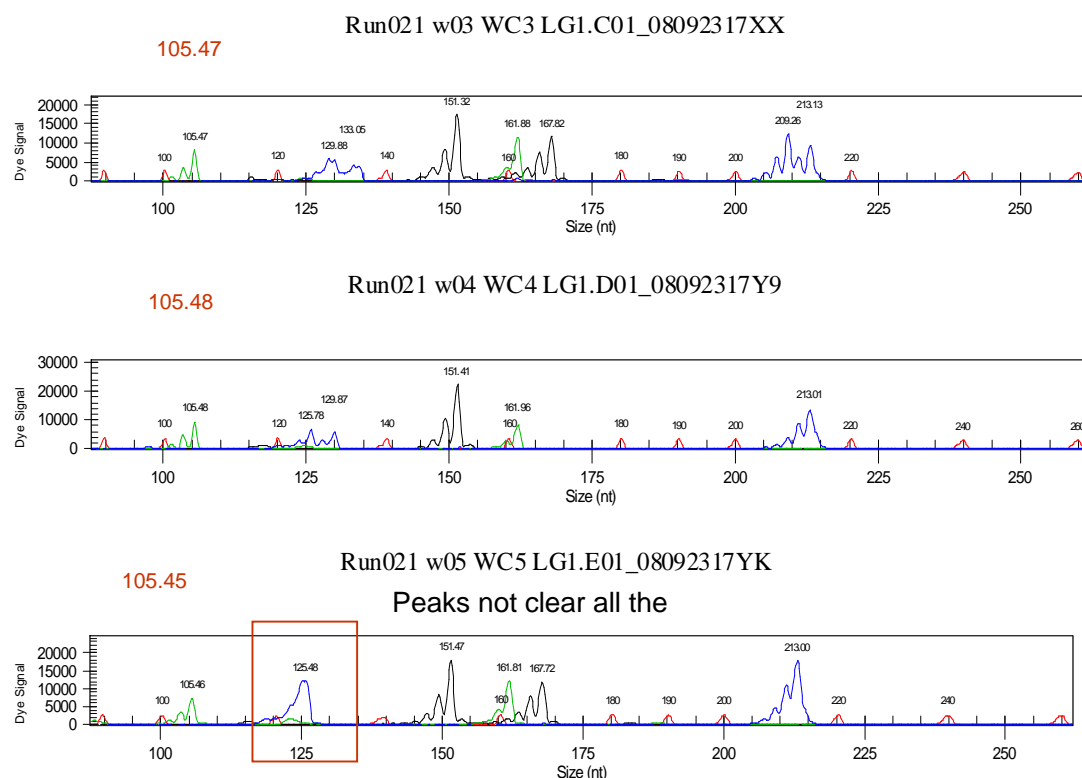


Figure 1. Beckman output for 6 different loci. Fluorescence reading for locus D08 are shown in read. An unclear peak is shown in a red square.

Once all the peaks were selected the sizes were plotted on a scatter graph to look for the ranges in size for each allele. Upper and lower limits for each allele were determined based on the distribution of the data in the graph (fig2). A corresponding 'character state' was given to each allele for further analysis.

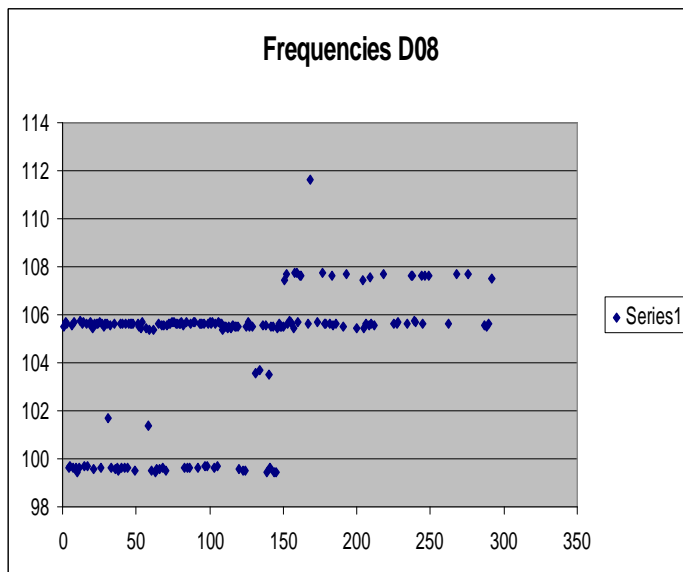


Figure 2. Scatter plot showing the distribution of the sizes from the sequencer for 6 different alleles.

For example for the data in fig. 2, I obtained six alleles with the following character states: 100, 102, 104, 106, 108 and 112. This way if the data was too spread and the ranges of each loci overlapped, the loci was eliminated from the analyses.

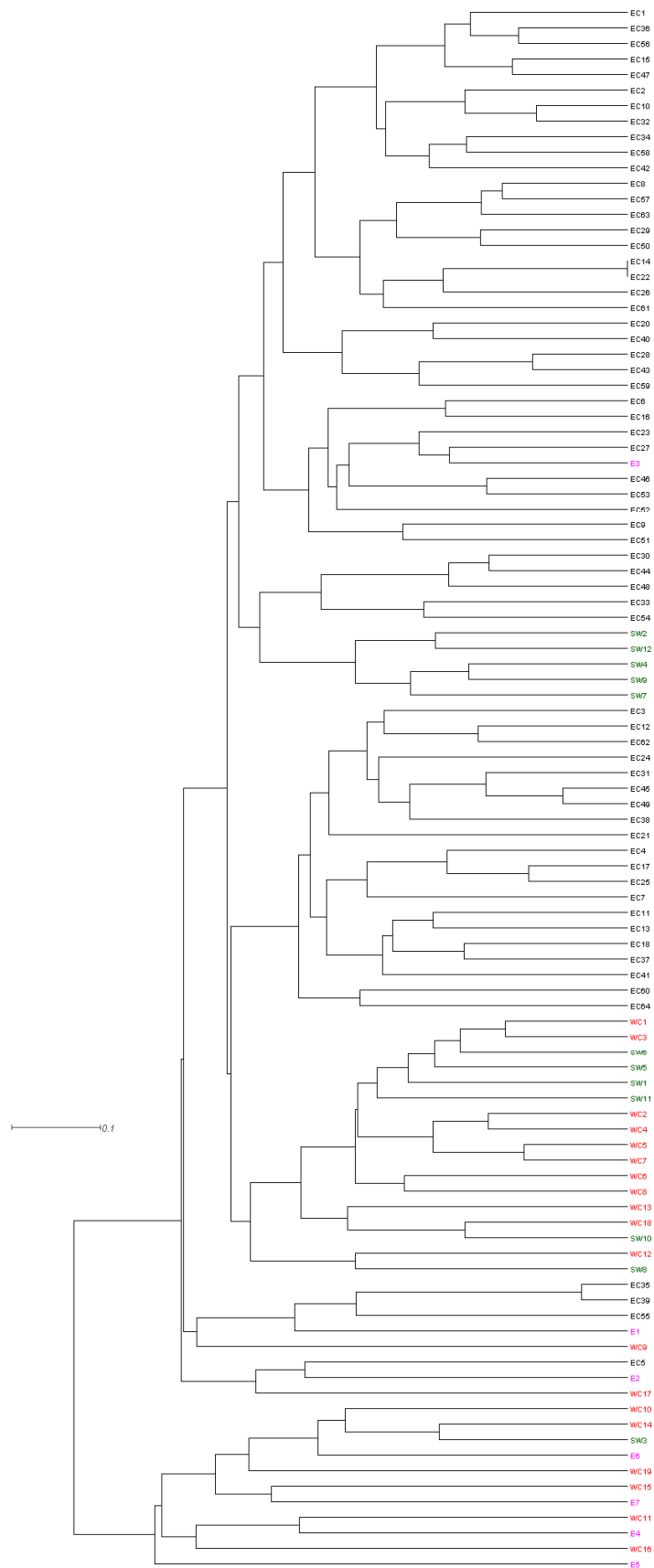
Appendix D Sequences obtained in Genbank

Accession numbers of sequences used for tree reconstructions:

Tt-PO_01 (DQ073704), Tt-PO_02 (DQ073714), Tt-PO-03 (DQ525385),
Tt-PO_04 (DQ525375.1), Tt-PO_05 (DQ525387), Tt-PO_06 (DQ073717),
Tt-PO_08(DQ525361), Tt-PO_09 (DQ073649), Tt-PO_10 (DQ073650),
Tt-PO_12 (DQ525380), Tt-PO_14 (DQ525384), Tt-PO_15 (DQ073655),
Tt-PO_16 (DQ525358), Tt-PO_17 (DQ525388), Tt-PO_20 (DQ525366),
Tt-PO_21 (DQ073684), Tt-PO_23 (DQ073696), Tt-PO_28 (DQ525386),
Tt-PO_31 (DQ525369), Tt-PO_32 (DQ525369), Tt-PO_33 (DQ525360),
Tt-PO_35 (DQ073716), Tt-PO_38 (DQ525360), Tt-PO_41 (DQ073681),
Tt-PO_43 (DQ525370), Tt-PO_48 (DQ073688), Tt-PO_53 (DQ073693),
Tt-PO_57 (DQ525387), Tt-PO_58 (DQ073700), Tt-PO_59 (DQ073699),
Tt-PO_61 (DQ073701), Tt-PO_65 (DQ073705), Tt-PO_67 (DQ073707),
Tt-PO_69 (DQ073709), Tt-PO_70 (DQ073710), Med1 (AY963604),
Med2AY96 (AY963603), Med3AY96 (AY963601), Med4AY96 (AY963598),
Med5AY96 (AY963596), Med6AY96 (AY963595), Med7AY96 (AY963594),
Med8AY96 (AY963602), Med9AY96 (AY963616), Med10AY9 (AY963614),
Med11Y96 (AY963612), Med12AY9 (AY963610), Med13AY9 (AY963609),
Med14AY9 (AY963608), Med15AY9 (AY963606), Med16AY9 (AY963605),
BSea1AY9 (AY963593), BSea2AY9 (AY963592), BSea3AY9 (AY963591),
BSea4AY9 (AY963590), BSea5AY9 (AY963589), ENA1AY96 (AY963621),
ENA2AY96 (AY963620), *Stenella coeruleoalba* (AY046542), *Grampus griseus*
(EU557095), *Orcinus orca* (DQ851148), *Orcinus orca* H13 (EU714135),
Tursiops aduncus (EF636212), *Tursiops aduncus*2 (EU557092),
Delphinus capensis (EU557094), *Sousa chinensis* (EU557091)

Appendix E UPGMA Complete tree

Relatedness Trees:



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