Phylogeography, population structure, abundance and habitat use of bottlenose dolphins, *Tursiops truncatus*, on the west coast of Ireland

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I declare that this thesis has not been previously submitted as an exercise for a degree at National University of Ireland, Cork, or any other university and I further declare that the work embodied in it is my own, or else noted.

Milaja Nykänen

Glossary of genetic terms used in the thesis

Allelic dropout: a source of missing data in microsatellite genotypes, in which one or both allelic copies at a locus fail to be amplified by the polymerase chain reaction.

Biogeography: the study of the distribution of species in geographic space and through (geological) time.

Cladogenesis: the formation of a new group of organisms or a higher taxon by evolutionary divergence from a "parent" taxon.

Coalescent theory: a stochastic model of population genetics that relates genetic diversity in a sample to demographic history of the population from which it was taken.

Complete lineage sorting: a perfect segregation of all alleles into all lineages.

DNA barcoding: a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species.

DNA library: a collection of DNA fragments.

DNA sequencing: the process of determining the order of nucleotides within a DNA molecule.

Ecotype: Here, defined as groups of populations that differ across geographic space in genetic (e.g. allele frequencies) and other (e.g. ecological, morphological, physiological) traits.

Effective population size (*Ne*): the number of individuals that an idealised population (with random mating, simultaneous birth of each generation, constant population size, equal number of offspring per parent) would need to have, in order for some specified quantity of interest to be the same in the idealised population as in the real population.

Fixation index (F_{ST} and F_{IS}): a measure of population or individual differentiation due to genetic structure (F_{ST}) or inbreeding (F_{IS}).

Founder effect: a non-random sampling that can exclude alleles from a new subpopulation by chance.

Founder effect: the loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population (a founder event).

Genetic drift: random sampling of allele frequencies in a population.

Haplotype: a group of genes in an organism that are inherited together from a single parent. A haplogroup is a group of similar haplotypes that share a common ancestor with a single nucleotide polymorphism mutation.

Hardy-Weinberg equilibrium (HWE): Principle stating that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors.

Incomplete lineage sorting: Under incomplete lineage sorting in population genetics, the coalescence time of genes to the same common ancestor and speciation time are different. Also referred to as deep coalescence when gene coalescence times are much older than species divergence times.

Introgression: Gene flow from one species into the gene pool of another by the repeated backcrossing of an interspecific hybrid with one of its parent species.

Linkage disequilibrium: the non-random association of alleles at different loci.

Microsatellite DNA: a sequence of repetitive DNA in which certain motifs (ranging in length from 2–5 base pairs) are repeated.

Models of DNA sequence evolution: To account for gene effects in the data set, each gene can be assigned a parameter that describes its substitution rate, e.g. GTR, HKY.

Multiplex polymerase chain reaction (Multiplex PCR): performing many separate PCR reactions all together in one reaction.

Null allele: a mutant copy of a gene at a locus that completely lacks that gene's normal function.

Operational Taxonomic Unit (OTU): DNA sequences can be clustered based on their similarity to one another according to similarity threshold set by the researcher.

PCR: Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA.

Phylogenetic tree, or **phylogeny**: a branching diagram displaying the inferred evolutionary relationships among taxons based upon similarities and differences in their physical or genetic characteristics.

Phylogeography: the joint phylogenetic relationships and geographic distributions of genetic lineages.

Population bottleneck: an abrupt reduction in population size due to environmental events or human activities.

Purifying selection: Many of the sequence polymorphisms that are seen among individuals of a population and in intraspecific comparisons are removed over evolutionary time due to the action of purifying selection or by random genetic drift.

Relaxed molecular clock: takes into account variability in substitution rates between lineages of the phylogeny.

Strict molecular clock: Nucleotide substitution rate model where the expected number of substitutions per year is constant regardless of which species' evolution is being examined.

Tajima's D: a statistic that compares the average number of pairwise differences with the number of segregating sites.

Time-dependency (in substitution rate estimation): Theory according to which nucleotide substitution rate estimates based on recent calibration points are much higher than those calibrated by older nodes.

Tree-branching models: the rate at which new branches are formed in a phylogenetic tree, e.g. Yule, birth-death model, coalescent model, etc.

Chapter 1: General Introduction

Bottlenose dolphins are among the most well-studied cetaceans in the world, with nearly 29,000 hits returned on Google Scholar using the search term "bottlenose dolphin". The early research on bottlenose dolphins concentrated largely on the natural history and distribution of the species (e.g. Miller 1923), and these were followed by studies on the morphology, physiology and behaviour, where data was generally collected by killing free ranging animals or capturing live animals for display and research purposes (e.g. True 1890; Gunter 1942; Fetcher & Fetcher 1942; McBride & Hebb 1948; Lilly & Miller 1961; Lilly 1962, 1963). What makes bottlenose dolphins such a well-studied species? One possible reason may lie within their acclaimed intelligence and their abilities to exhibit complex social structures and behaviours otherwise found in the animal kingdom only within other delphinids, primates and elephants (e.g. Reiss & Marino 2000; Janik 2000; Connor 2000; Connor 2007; Möller *et al.* 2012). Other reasons for the appeal of this species among scientists may be their world-wide distribution (Fig. 1.1) and the fact that the proximity of some coastal bottlenose dolphin populations to human settlements makes them more available as a study species compared to some more elusive or hard to reach cetaceans. At the same time this proximity to land also makes them more vulnerable to human impacts – a key reason why coastal populations have become the focus of conservation efforts, requiring management and monitoring strategies to be put to place.

The fact that they spend their lives under water makes studying wild bottlenose dolphin populations difficult and presents several challenges as the data collection is usually constrained to good weather and light conditions. Nevertheless, the fact that individual dolphins can often be distinguished from unique sets of markings accumulated on their dorsal fins or bodies (e.g. Würsig & Würsig 1977; Würsig & Jefferson 1990), makes it possible to gather information on their abundance, movements and social structure through mark-recapture techniques. The methods relating to these topics are discussed more thoroughly in Chapters 3 and 4. However, whilst the resident coastal populations can be accessed relatively easily by using small vessels and sometimes even from land, collecting data on coastal individuals or populations that exhibit more unpredictable ranging patterns or whose distribution is concentrated to areas further offshore, presents even further challenges. In situations like these, alternative strategies, such as

the genetic sampling of stranded or bycaught individuals, can provide a way for gathering information on these populations (Chapters 2 and 3). In mark-recapture abundance estimation, on the other hand, alternative modelling approaches that allow for more flexible survey schemes can provide efficient and cost-effective alternatives to traditional models (Chapter 4). Furthermore, using a combination of different methodologies, such as a passive acoustic monitoring (PAM, Chapter 5) coupled with visual monitoring methods, can provide a way of monitoring habitat use of bottlenose dolphins and may even be used to direct (visual) survey effort into an appropriate area and season.

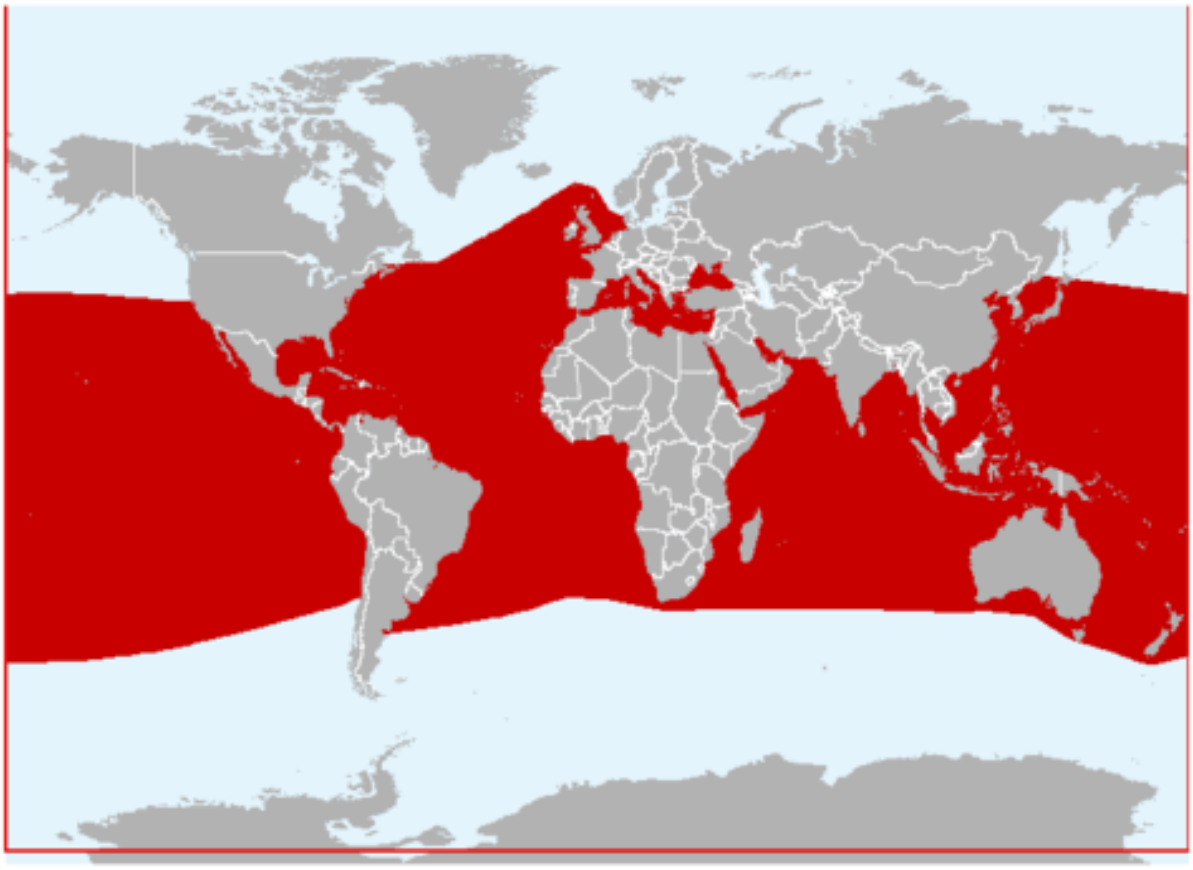

Figure 1.1 Map showing the world-wide distribution of *Tursiops truncatus*. With the exception of polar regions, the species is found throughout the world's oceans. (IUCN 2012).

1.1 Taxonomy and phylogenetics of bottlenose dolphins

In short, the purpose of taxonomy is to provide a way to organise and summarise information on the relationships of organisms and to divide them into hierarchical

classes based on similarities and dissimilarities. This kind of classification can also reveal information on the evolutionary pathways along which present-day organisms may have developed from ancestral forms. Before the introduction of molecular genetics and DNA sequencing technologies, studies resolving the taxonomy of organisms within the family Delphinidae were primarily done by careful examination and measurement of morphological features, with particular focus on the morphology of the skull (reviewed by LeDuc *et al.* 1999). Even today morphometric methods are used to support the phylogenetic relationships within the delphinid family (e.g. Amaral *et al.* 2009). Recently, methods that combine information on fossil morphometrics and genetic data into 'total-evidence dating' phylogenetic models have been developed (Ronquist *et al.* 2012).

Due to its clock-like nucleotide substitution rate and the fact that it is expected to conserve and accumulate mutations over time (Ho *et al*. 2008), the reconstruction of the phylogenetic history of organisms has primarily been done by sequencing parts of the maternally inherited mitochondrial genome. The first studies resolving the phylogenetic history of delphinids used only short sequences of the genome, such as the control region and cytochrome b gene in the genus *Delphinus* (Rosel *et al.* 1994) and family Phocoenidae (Rosel *et al.* 1995), and at a wider taxonomic level of the subfamily Delphininae (LeDuc *et al.* 1999), but during recent years, the emergence of Next Generation Sequencing (NGS) technologies has vastly increased the amount of genetic data that can be generated for phylogenetic studies. This combined with the development of phylogenetic statistical tools (see review by Yang & Rannala 2013), has improved the ability to find phylogenetic differences and increased the resolution in species divergence estimation. For example, Steeman *et al.* (2009) used six mitochondrial and nine nuclear genes combined with fossil calibrations to reconstruct the phylogenetic history of cetaceans (Fig. 1.2), and within the same year, a study by McGowen *et al.* (2009) used an impressive set of mitochondrial genomes and nuclear genes to build a comprehensive, time-calibrated phylogenetic tree of the same taxonomic group.

Previous phylogenetic studies, including the two mentioned above, have all suggested a relatively recent (beginning ~10 million years ago) and rapid radiation within Delphinidae (Steeman *et al.* 2009; McGowen *et al.* 2009; Kingston *et al.* 2009; Xiong *et al.* 2009), hypothesized to be linked to periods of environmental fluctuations

(McGowen *et al*. 2009, Steeman *et al*. 2009: Vilstrup *et al*. 2011), which has led to uncertainties in the phylogenetic relationships within the family. Vilstrup *et al.* (2011) aimed to resolve these uncertainties by increasing the amount of mitogenome sequences from this family, including several sequences from within the same species, followed by Amaral *et al.* (2012) who constructed a species tree from both mitochondrial and nuclear data using coalescent analysis (Fig. 1.3).

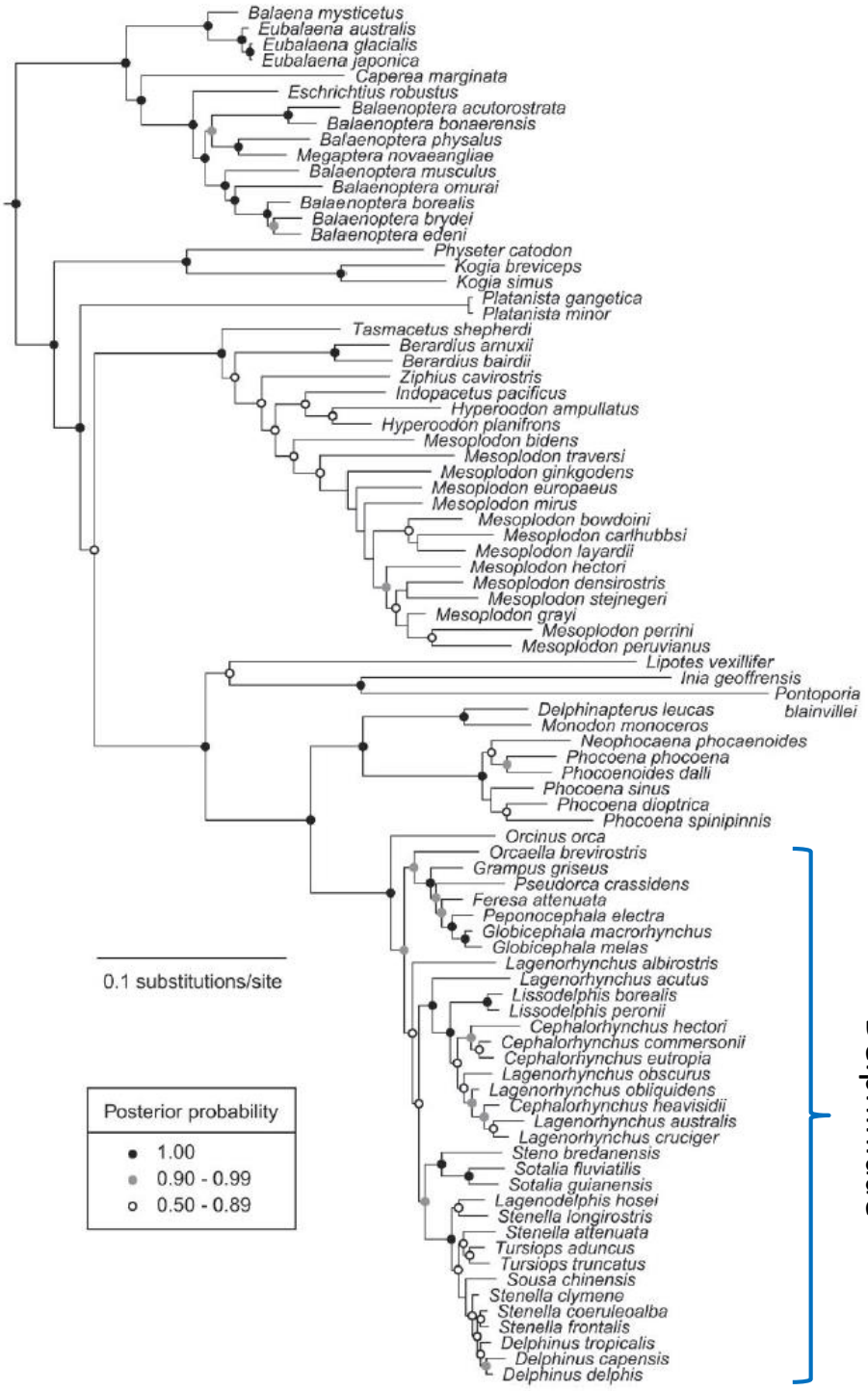

Figure 1.2 Phylogenetic tree of the order Cetacea taken from Steeman *et al.* (2009). Note the rapid radiation observed in the family Delphinidae.

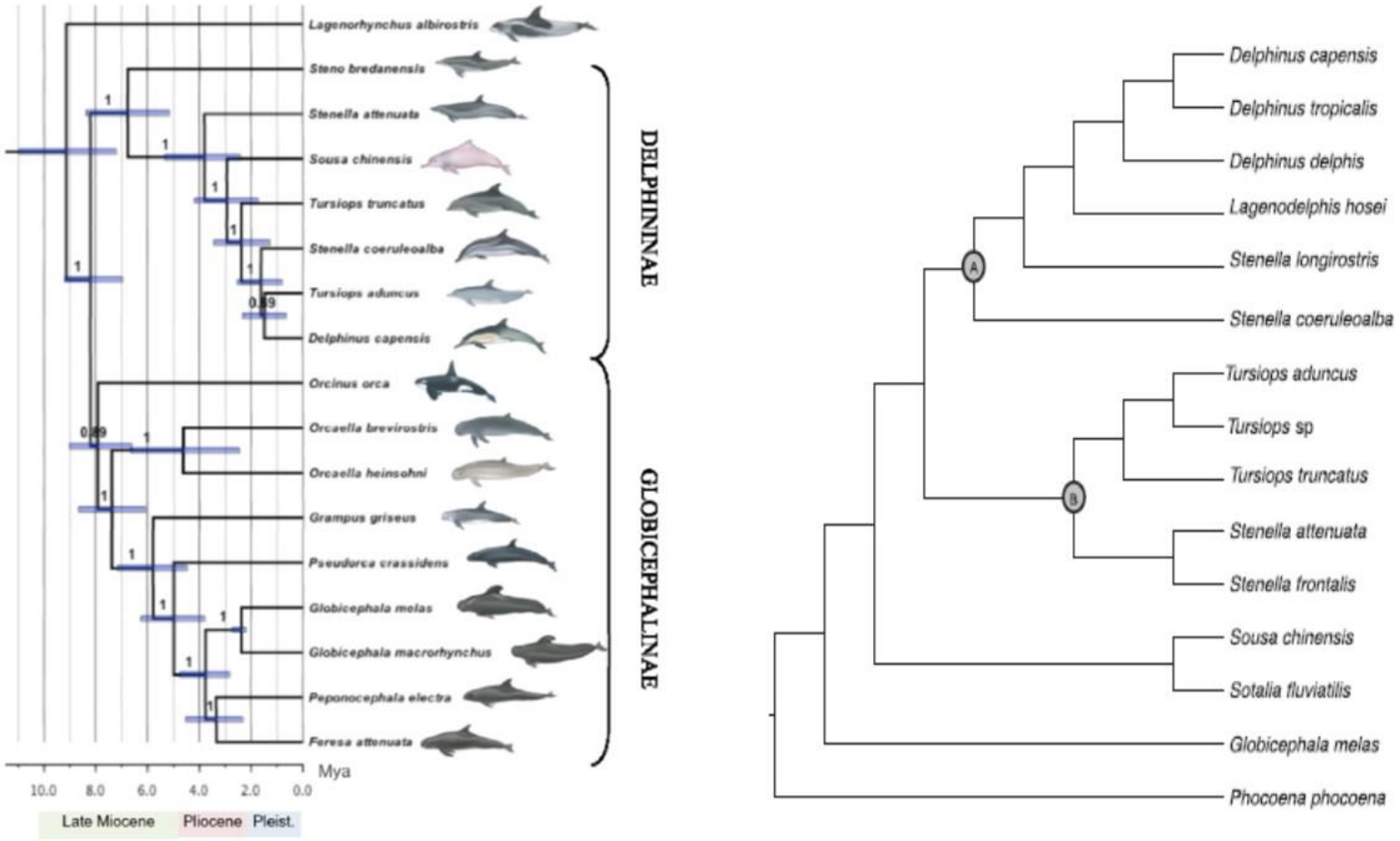

Figure 1.3 Phylogenetic trees of the superfamily Delphinoidea taken from Vilstrup *et al.* (2011) (on the left) and from Amaral *et al.* (2012) (on the right).

The taxonomy within the genus *Tursiops* is similarly unclear, and depending on the DNA markers and phylogenetic method used, contradicting topologies have been produced (LeDuc *et al.* 1999, Wang *et al.* 1999; Vilstrup *et al.* 2011; Amaral *et al.* 2012). In addition to the common bottlenose dolphin, *Tursiops truncatus* (Montagu 1821), Indo-Pacific bottlenose dolphin, *Tursiops aduncus,* has been recognized as a separate species by a number of international organisations, including the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) based on phylogenetic and morphological evidence (Curry & Smith 1997; LeDuc *et al.* 1999, Wang *et al.* 1999). This was followed by another species designation of *T. australis*, the Burrunan dolphin, based on differences in morphology, colouration, cranial characters and genetic evidence (Charlton-Robb *et al.* 2011). However, the validity of the latter species is still subject to debate and not yet fully recognised (Committee on Taxonomy 2014).

Following a debate within CITES whether a putative Black Sea subspecies of bottlenose dolphin, *Tursiops truncatus ponticus*, should be given a full species status, a specialised Workshop on Cetacean Systematics was held to review cetacean taxonomy (Reeves *et al.* 2004). As a result of this workshop, it was decided that for species delineation, both morphological and genetic data would have to be consistent to prove "irreversible reproductive isolation" (Reeves *et al.* 2004). A key question is thus whether the genus *Tursiops* should undergo further division with the separation of the two forms (or 'ecotypes') in the Western North Atlantic, an offshore and a coastal form based on morphology and ecological and genetic markers (Duffield *et al.* 1983; Hersh & Duffield 1990; Mead & Potter 1995; Leduc & Curry 1997, Hoelzel *et al.* 1998).

1.2 Biogeography

Moura *et al.* (2013) analysed an impressive set of complete mitochondrial genomes, which included samples collected from Australia, Africa, North Pacific, North Atlantic, Mediterranean and Black Sea. The results suggested that the extant members of the genus *Tursiops* originated from Australasia from lineages occupying coastal habitats. The authors also hypothesised that the expansion of the lineages to the

Atlantic occurred via Indo-Pacific coastal habitats followed by the colonization of the pelagic area and the delineation of the *T. truncatus* species. *T. truncatus* then regressed back to the ancestral coastal state and are represented, among others, by the coastal populations on the West Coast of North America (Moura *et al.* 2013).

1.3 Distribution and habitat use

Resources in natural ecosystems are variable and usually patchily distributed in both time and space. It is thus not surprising that most marine predators are not randomly or uniformly distributed, but that their distribution can be driven by a combination of abiotic or biotic factors, such as depth, temperature, ocean currents and fronts, benthic type, bathymetry, prey distribution, presence of predators or conspecifics and anthropogenic factors (e.g. Ford *et al.* 1998; Saulitis *et al.* 2000; Heithaus & Dill 2002; Ingram & Rogan 2002; Johnston *et al.* 2002; Olesiuk *et al.* 2002; Williams *et al.* 2002; Embling *et al.* 2010; Sveegard *et al.* 2012; Booth *et al.* 2013; Pirotta *et al.* 2011; Scales *et al.* 2014). Bottlenose dolphins are top marine predators with a world-wide distribution extending from tropical to temperate waters (Leatherwood and Reeves 1990; Connor *et al.* 2000, see Fig. 1.1). Fine-scale habitat use of bottlenose dolphins has been linked to a variety factors. For example, Wilson *et al.* (1997) found that dolphins in the Moray Firth, Scotland, occurred mostly in areas with deep channels associated with strong tidal currents. Bottlenose dolphins were present in the bay year-round but the use of different parts of the bay changed seasonally with the number of individuals increasing in the summer months (Wilson *et al.* 1997) and during flood tides (Mendes *et al.* 2002). Further, video surveillance in these channels revealed that the dolphins were seen most frequently within the deepest waters and areas of steep seabed gradients (Hastie *et al.* 2003). Subsequently, Hastie *et al.* (2004) found evidence that the dolphins were engaged in feeding behaviour in these high use areas, with a correlation between the occurrence of feeding and increasing bathymetry (Hastie *et al.* 2004). Within the Shannon estuary, Ireland, bottlenose dolphins were also encountered in deeper areas with steep sloping benthic topography rather than in shallower areas with lower benthic gradients, even though individual differences existed in habitat use (Ingram & Rogan 2002).

In environments with less local variation caused by benthic topography, such as the open ocean pelagic zone, seasonal effects can have a stronger influence on habitat use of marine mammals. For example, Bearzi *et al.* (2008) found several environmental covariates explaining the presence of bottlenose dolphins but the effect varied depending on the season.

In addition to the distribution of resources and conspecifics, the presence of predators can have an effect on the habitat use of animals. Indo-Pacific bottlenose dolphins (*T. aduncus*) in Shark Bay, western Australia were found to utilize the food-rich shallow habitats more during winter months when predation risk from tiger sharks (*Galeocerdo cuvier*) was lower, moving towards less productive deeper areas for summer months when the predation risk was increased (Heithaus & Dill 2002). In addition, the distribution of dolphins was found to be approximately proportional to prey density when shark abundance was low but at high shark abundance the dolphin distribution changed towards safer, deeper, but less productive waters (Heithaus & Dill 2002). The presence of another shark predator, bull shark (*Carcharhinus leucas)*, during the summer months in the inshore waters of the Gulf of Mexico may have driven the distribution of bottlenose dolphins into protected bays (Wells & Scott 2002).

Anthropogenic disturbance can have an effect on the habitat use of marine mammals and it can even cause permanent displacement from an area. In New Zealand, for example, bottlenose dolphins have been encountered less in Milford Sound, as a result of increased boat traffic (Lusseau 2005). Similarly, the abundance of Indo-Pacific bottlenose dolphins in Shark Bay has decreased in areas with dolphin-watching boats (Bejder *et al.* 2006). Kuningas *et al.* (2013) found the presence of killer whales, *Orcinus orca*, to be negatively affected by naval sonar activity but this effect was masked by herring (*Clupea harengus*) abundance, the main factor affecting killer whale presence in that area. Further, Miller *et al.* (2015) found naval sonar signals to affect the diving and echolocation behaviour of northern bottlenose whales *Hyperoodon ampullatus*; the animals ceased their echolocation during the exercise and one animal that was tracked avoided the sound source by changing direction and swimming away from it.

1.4 Social structure

Bottlenose dolphins generally live in fluid "fission-fusion" societies (Connor *et al.* 2000), which means that dolphins usually form small social groups whose composition can change rapidly within the scale of a few hours (see page 109 in Connor *et al.* 2000). The fluidity of dolphin social networks has recently been linked to the low cost of locomotion (Randić *et al.* 2012). Gender can also influence the associations of dolphins, however, this does not apply to every population. In Shark Bay, Australia, Smolker *et al.* (1992) observed long term associations in *Tursiops* spp, generally between members of the same sex with the strongest associations between a mother and her offspring or between two males. These first-order male subgroups in turn had moderate associations with other male first-order subgroups thus forming second-order alliances. Similarly, females preferred to associate with certain other females forming a network where all females were interconnected through a chain of consistent associates, but in general, the associations were less stable (Smolker *et al.* 1992). Connor *et al.* (2000) hypothesized that the male-male alliances in Shark Bay are formed due to the need for co-operation in herding females. The social structure and stability of these first-, second- and even third-order alliances in Shark Bay have recently been re-examined and reviewed (Connor *et al.* 2011; Connor & Krützen 2015), and the results have suggested that second order alliances may persist for up to 20 years, highlighting the importance of long term studies in understanding dolphin societies. These stable alliances may be formed in order to increase individuals' inclusive fitness by kin selection; for example, Krützen *et al.* (2003) found that the males in stable first- and second-order alliances were often strongly related. Similarly, Frere *et al.* (2010a) found that female bottlenose dolphins preferred to associate with closely related individuals than expected by chance. Close social bonds have been found to correlate with increased calving success thus promoting individual's fitness (Frere *et al.* 2010b).

Strong male-male associations between bottlenose dolphins were also observed in Sarasota, Florida, but co-operative second-order alliances were not recorded (Connor *et al.* 2000). In contrast, no strong sex-specific alliances were found in the Moray Firth (Wilson 1995) or in the Shannon estuary in Ireland (Foley *et al.* 2010), and this has been suggested to be caused by local site-specific differences in resource competition and/or the reduced risk of predation (Wilson 1995). Sex-specific alliances were not

observed amongst the dolphins in Doubtful Sound, New Zealand, but some male–female associations were stable over the course of several years (Lusseau *et al.* 2003).

Specialised foraging techniques, which may promote the formation of social groups and thus have important ecological and evolutionary consequences contributing to niche specialisation and possibly even to genetic structuring (Krützen *et al.* 2014; Kopps *et al.* 2014), have also been observed in bottlenose dolphin communities (Mann & Sargeant 2003). For example, some social groups in Shark Bay are known for their tool use, i.e., "sponging" (Smolker *et al.* 1997; Mann *et al.* 2008; Krützen *et al.* 2005, 2014) and to a lesser extent, "conching" (Allen *et al.* 2011). Strand feeding (e.g. Duffy-Echevarria *et al.* 2008; Jimenez *et al.* 2015) and beach hunting (Sargeant *et al.* 2005) have been observed in dolphin communities in different parts of the world along with feeding associated with trawlers (e.g. Broadhurst 1998; Chilvers & Corkeron 2001; Gonzalvo *et al.* 2008). Interestingly, Chilvers and Corkeron (2001) found dolphins in Moreton Bay, Australia, feeding from trawling nets to almost exclusively associate with other "trawler feeders" thus forming a separate community from other individuals who did not use this foraging tactic. However, this social separation disappeared when trawling was banned in the bay, being replaced by a more fluid social structure with more associations occurring between all the dolphins (Ansmann *et al.* 2012), which indicates that social clusters can be fluid and may form as a response to a newly available resource and dissipate when this resource is removed. Another example of social groups forming based on specialized foraging techniques include the bottlenose dolphins in Laguna, Brazil, where certain bottlenose dolphins have learned to co-operate with fishermen, seemingly driving mullet (*Mugil* spp.) into their nets and catching the escaping fish (Simões-Lopes *et al.* 1998; Daura-Jorge *et al.* 2012).

1.5 Population structure of bottlenose dolphins in the North Atlantic Ocean

The lack of physical barriers in the marine environment combined with the fact that many marine organisms have good capabilities for dispersal, should, in theory, mean high gene flow and lack of speciation in marine organisms. This is the case with most marine species, as very little genetic differentiation is usually observed even with distances of thousands of kilometres (reviewed by Palumbi 1994). In highly mobile organisms, such as birds and mammals, gene flow is often reduced due to socio-

ecological, behavioural and/or environmental factors that can lead to divergence of populations. For example, female philopatry and sex-biased dispersal, combined with short dispersal distances shown by the less philopatric males may have led to fine-scale population structuring of co-operatively breeding birds (Temple *et al.* 2006). Similarly, long-term fidelity to natal social clusters shown by both sexes, possibly enforced by differences in foraging strategies required to exploit resources in varying environments are thought to have led to fine-scale population structuring of two resident populations of false killer whales (*Pseudorca crassidens*) around Hawaii (Martien *et al.* 2014). Wolf *et al.* (2008) also found natal philopatry among breeding female Galápagos sea lions *(Zalophus wollebaeki)* and hypothesized habitat specialization as one of the driving forces behind genetic divergence. High levels of relatedness between close associates may have further driven fine-scale population structuring of some species (e.g. Temple *et al.* 2006; Iacchei *et al.* 2013), even when accounting for spatial proximity (Podgórski *et al.* 2014), thus reinforcing interactions among related individuals and encouraging philopatry especially when food sources are limited or patchy. Similarly, many marine organisms show population structure over small geographic scales (see review by Palumbi 1994; Bierne *et al.* 2003). This can lead to severe loss of heterozygosity over time due to genetic drift and inbreeding, especially if populations are small and isolated (Lacy 1987). Possible drivers of this fine-scale population structure include existence of ocean fronts (White *et al.* 2010), historic and oceanographic influences (Woodall *et al.* 2015), isolation by distance, historical founding events, complex social interactions, natal philopatry, and development of foraging specializations and habitat preference possibly leading to adaptive isolation (e.g. Natoli *et al.* 2005; Krützen *et al.* 2004; Rosel *et al.* 2009). In a recent review, Möller (2012) suggests that environment type often dictates the social bonds observed in populations, with more female-biased philopatry found among small delphinids inhabiting shallow inshore environments with more predictable resources when compared to those inhabiting offshore deeper environments where food availability is less predictable.

The bottlenose dolphin shows hierarchical population structure throughout its world-wide range, with the greatest divergence found between pelagic and coastal populations (Curry & Smith 1997; Hoelzel *et al.* 1998; Louis *et al.* 2014a; Lowther-Thieleking *et al.* 2015). This is often accompanied by ecological and/or morphological

differences (Duffield *et al*. 1983; Hersh & Duffield 1990; Hoelzel *et al.* 1998; Louis *et al.* 2014a; Natoli *et al.* 2004) and a division to offshore and inshore forms, or *ecotypes*, has been suggested based on these differences in the Northwest Atlantic (Duffield *et al*. 1983; Mead & Potter 1995) and more recently, also in the Northeast Atlantic (Louis *et al*. 2014a). Moreover, Hoelzel *et al.* (1998) analysed nuclear microsatellites from nearshore and offshore bottlenose dolphins from the western North Atlantic (WNA), the Bahamas and Africa and found that the level of genetic variation among the nearshore dolphins was reduced compared with the offshore population in the WNA. Greater genetic diversity among the North Atlantic offshore bottlenose dolphins has since been found in other studies (e.g. Natoli *et al.* 2004; Tezanos-Pinto *et al.* 2009; Mirimin *et al.* 2011; Louis *et al.* 2014a, see also Chapters 2 and 3). However, the division to inshore and offshore forms may not necessarily apply in other parts of the world; Hoelzel *et al.* (1998) found all the nearshore African haplotypes to cluster together with the WNA offshore population.

Parsons *et al.* (2002) compared microsatellite markers sampled from bottlenose dolphins around the UK and found significant levels of genetic differentiation between the northeast of Scotland (the Moray Firth) population and the west coast of Scotland populations. In fact, the NE Scotland dolphins appeared to be more closely related to the bottlenose dolphins occupying Cardigan Bay, Wales, than to the ones found in the Sound of Barra on the Scottish west coast, despite the larger geographic distance (Parsons *et al.* 2002). Mirimin *et al.* (2011) analysed biopsy samples from bottlenose dolphins along the west coast of Ireland and found that the samples collected in Connemara (Co. Galway) and Mayo (Co. Mayo) belong to the same genetic population. These samples differed significantly from samples collected in the Shannon estuary (~150km south of Connemara) with both populations showing low genetic diversity (Mirimin *et al.* 2011). However, most samples collected from stranded dolphins along the Irish west coast showed much greater genetic diversity compared to the coastal populations, and Mirimin *et al.* (2011) hypothesised that these samples likely originated from a third putative offshore population inhabiting the waters between the coast and continental shelf. The dolphins using the coastal waters of western Ireland appear to be also socially distinct from dolphins using offshore waters (Oudejans *et al.* 2015).

Nichols *et al.* (2007) suggested that coastal bottlenose dolphins in the NE Atlantic may be part of a wider meta-population based on reduced, but ongoing gene flow among small local populations that seem to be dependent on local habitat patches (Natoli *et al.* 2005; Sellas *et al.* 2005), and this was further supported by Louis *et al.* (2014a) whose study clustered all the coastal samples collected in Ireland and the UK into a single 'Coastal North' population. In a further study, also based on neutral genetic markers, Louis *et al.* (2014b) suggested that the broad-scale division to 'coastal' and 'pelagic' ecotypes in the NE Atlantic may reflect a historical divergence of populations followed by colonisation events of the coastal populations into available inshore habitats from an oceanic source population after the Last Glacial Maximum, LGM (Louis *et al.* 2014b). Divergence between the 'Coastal North' and the 'Pelagic Atlantic' populations was estimated to date to ca. 10,320 years before present (yBP) and the colonisation was suggested to have occurred via a small number of founding individuals (Louis *et al.* 2014b). The end of the last glacial period in the Northern Hemisphere is thought to have had a major impact on genetic diversity of organisms (Hewitt 1999, 2000), and post-glacial colonisations such as the above would have been a common pattern during post-glacial periods, leaving a genetic signature in present day populations (Hewitt 2000). Furthermore, Moura *et al.* (2013) found the differentiation of the coastal and pelagic WNA ecotypes coinciding with periods of fast climatic change during the Eemian and Holocene periods, and suggested that climatic oscillations may be involved in the diversification of bottlenose dolphins in this area (Moura *et al.* 2013). Conversely, Louis *et al.* (2014b) concluded that the divergence of the 'Coastal North' and 'Coastal South' populations (ca. 2,560 yBP) was not linked to any particular climatic event.

1.6 The use of photo-identification and mark-recapture methods in the study of bottlenose dolphins

Understanding the distribution, movements and abundance of cetacean populations currently relies largely on our ability to recognise individuals from unique markings. Photo-identification is a technique used to identify individual animals from photographs using naturally occurring markings as distinctive characters (see Fig. 4.2 in Chapter 4). This method was first used to identify individual bottlenose dolphins by Caldwell (1955), Irvine & Wells (1972) and Würsig & Würsig (1977), since most

bottlenose dolphins accumulate distinctive notches and scars on their dorsal fins and bodies through social interactions with conspecifics.

Mark-recapture, or capture-recapture methods in abundance estimation of closed and open populations are mostly different variations of the Petersen estimator (Seber 1982, 1992). With 'population closure' it is assumed that the population is stable, with net migration and the difference between births and deaths both equalling zero; needless to say that this assumption is rarely fulfilled in natural populations. The Petersen estimator, $\widehat{N}$, is obtained by equating the proportion of initially marked and released individuals (m_1) to the total number of animals in the population (N) with the proportion of marked animals (m_2) to the number of unmarked and marked animals in the second sample (n_2)

$$\widehat{N} = \frac{m1n2}{m2}$$

If more than two samples are collected, the unmarked animals can be marked in the second sample and subsequently all the animals released again. This sequential mark and release process will lead to individual capture histories (reviewed by Schwartz & Seber 1999).

Mark-recapture abundance models are inevitably associated with uncertainty resulting from heterogeneity in the capture, survival and sighting probabilities (Scwartz & Seber 1999), and several modifications to the models have been developed to quantify this heterogeneity (e.g. Seber 1992). Possibly the most frequently used method to estimate bottlenose dolphin abundance has been M_{th} by Chao *et al.* (1992) due to its good performance in abundance estimation of populations with reasonable amounts of heterogeneity. Log-linear models (Fienberg 1972) are also particularly useful for modelling both heterogeneity in capture probabilities and their dependencies between sampling occasions. In general, due to the wide variety of available mark-recapture models, the choice for the most suitable model should ideally depend on the characteristics of the data collected (Durban *et al.* 2005).

Bayesian statistics, as opposed to traditional frequentist Maximum Likelihood (ML) based estimation, have recently become more commonly used in mark-recapture abundance estimation (e.g. Mäntyniemi & Romakkaniemi 2002; Michielsens *et al.* 2006), and they have especially been applied to abundance estimation of a variety of

cetacean species (e.g. Durban et al 2005; Durban *et al.* 2010; Moore & Barlow 2011; Fearnbach *et al.* 2012; Cheney *et al.* 2013). Bayesian methods are particularly useful when data are sparse (e.g. Durban *et al.* 2005; Royle *et al.* 2007; Cheney *et al.* 2013) due to the possibility of using Monte Carlo methods to sample from the posterior distribution (Scwartz & Seber 1999). This makes them highly applicable to cetacean abundance studies that are often burdened by small data sets due to challenges involved in data collection resulting from, for example, poor weather conditions or unpredictable occurrence and elusive behaviour of the study species.

1.7 Conservation and management of bottlenose dolphins

On a global scale, common bottlenose dolphins, are considered as of 'least concern' by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Hammond *et al.* 2012). Subspecies *T. truncatus ponticus* in the Black Sea is listed as 'endangered' and the *T. truncatus* populations occurring in the Mediterranean Sea 'vulnerable' (Birkun 2012; Bearzi *et al.* 2012). Bottlenose dolphins are also listed in Annex II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which prohibits their commercial trade in countries that have signed the treaty.

In European waters, bottlenose dolphins are protected through the Habitats Directive (92/43/EEC), and like all cetaceans, they are listed in Annex IV of the Habitats Directive necessitating 'strict protection' for such species. In addition, bottlenose dolphins are listed in Annex II of the Habitats Directive; this means that the Member States are required to designate Special Areas of Conservation (SACs) as part of a European strategy to maintain or restore a favourable conservation status for the species (Natura 2000). Further, as top predators, bottlenose dolphins are considered as one of the indicator species for 'good environmental status' in coastal waters in the Marine Strategy Framework Directive (MSFD, 2008/56/EC). The aim of the MSFD is to protect the European marine environment by applying a wholesome ecosystem-based approach to the management of human activities in these waters whilst encouraging sustainable use of the environment.

Despite the lack of consensus among researchers on what constitutes a population (Waples & Gaggiotti 2006), defining populations either as Management Units (MUs)

or Evolutionarily Significant Units (ESUs) (Moritz 1994), is relevant when setting strategies for conservation and management. Moritz (1994) defined MUs as "population units with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles". ESUs, on the other hand, were defined by Moritz (1994, 2002) as units arising from "historical population structure rather than current adaptation that are reciprocally monophyletic for mitochondrial DNA and show significant divergence of allele frequencies at nuclear loci". Crandall *et al.* (2000) added the importance of ecological exchangeability to the equation stating that "the rejection of, or failure to reject, (genetic or ecological) exchangeability forms the foundation of population distinctiveness and management practices". However, Waples and Gaggiotti (2006) and Palsbøll *et al.* (2006) criticised the use of the statistical criterion in these definitions stating that population structure may go undetected when population genetic divergence is low and has occurred relatively recently, since the statistical power to detect population structure is a function of the amount of data and thus correlates to the number of loci used and samples analysed. In these cases, using 'kinship-based' approaches and/or other methods in quantifying recent (past few generations) migration among putative populations may offer a solution to the delineation of MUs (Waples & Gaggiotti 2006; Palsbøll *et al.* 2010).

Bottlenose dolphin populations using coastal environments are at particular risk of exposure to a number of anthropogenic threats which may directly impact individuals, for example through disturbance or damage to health and to the overall functioning of the coastal ecosystems upon which they depend. The sensitivity of bottlenose dolphins to these threats is exacerbated by their position as an apex predator and also by their low reproductive rates (Connor *et al.* 2000; Quick *et al.* 2014). The main threats in coastal environments include pollutants such as xenobiotic chemicals (especially PCBs and DDTs) (Jepson *et al.* 2016), reduced prey availability, habitat degradation, disturbance from vessel traffic (Lusseau *et al.* 2009; Williams *et al.* 2009; Pirotta *et al* 2015), entanglement and incidental bycatch, direct hunting, marine construction and anthropogenic noise (Hammond *et al.* 2012; Meissner *et al.* 2015; Pirotta *et al.* 2015). The determination of impacts from anthropogenic habitat degradation on coastal populations requires detailed understanding of the population structure and connectivity so that defining appropriate Management Units (MUs) or Evolutionarily

Significant Units (ESUs) can be achieved and the size of the communities/populations facing these threats estimated.

1.8 Background to the project and main aims of the study

Most research effort on bottlenose dolphins in Ireland has largely concentrated on dolphins using the Shannon estuary whose abundance has been estimated as approximately 140 individuals (Ingram & Rogan 2002; Berrow *et al.* 1996, 2012; Ingram & Rogan 2003; Englund *et al.* 2007, 2008; Rogan *et al.* 2015). In the early 2000s, the lower part of the estuary was designated as a SAC to ensure the protection for this population (Lower River Shannon SAC, see Fig. 1.4). However, in addition to the Shannon population, bottlenose dolphins range widely on a large part of the west coast of Ireland. Photo-identification surveys targeting these west coast animals commenced in 2001 when Ingram *et al.* (2001) completed surveys in Brandon Bay, (Co. Kerry), Connemara (Co. Galway), Broadhaven Bay (Co. Mayo) and McSwyne's Bay (Co. Donegal). High number of individuals were sighted during these surveys and further genetic work suggested that these mobile animals using the coastal habitats in western Ireland belong to a genetically distinct population separate from the dolphins occupying the Shannon estuary (Mirimin *et al.* 2011). In 2009, an abundance estimate of 171 was derived from dedicated survey data collected during surveys of north Connemara waters (Ingram *et al.* 2009). This estimate represented the first attempt to assess the number of animals using a site outside of the Lower River Shannon SAC, and combined with the distribution of sightings during previous survey work and the genetic results from Mirimin *et al.* (2011), led to the designation of a second SAC for bottlenose dolphins on the Irish west coast in 2013 (West Connacht Coast SAC). This SAC roughly covers areas in the northern parts of Connemara, and west of the Mullet Peninsula, Co. Mayo (see Fig. 1.4). However, the number of genetic samples acquired from this area was quite low (representing less than 10% of the population), therefore it remained unclear whether even finer population structure exists among these dolphins. Moreover, the abundance surveys in 2009 were restricted to a relatively small area in Connemara, and from the comparison of photographed animals to the photo-identification archives collected over the years it was apparent that the animals were ranging well beyond this area with matches of individuals as far apart as Co. Cork and Co. Donegal. Therefore, one of the aims of this PhD thesis was to re-

investigate the population structure of bottlenose dolphins in Irish waters by analysing more genetic samples collected in a wider coastal area outside the Shannon estuary and from stranded individuals.

Figure 1.4 Special Areas of Conservation (SACs) designated for bottlenose dolphins, *Tursiops truncatus,* in Irish waters.

As detailed earlier, and especially in light of the MSFD and the recent SAC designation for the transient coastal population in Irish waters, it is essential to set up a monitoring strategy for these animals, and the first step towards an efficient monitoring plan

involves the identification of Management Units (MUs). The first and second data chapters of this thesis address this issue, first by investigating the historical divergence and colonisation patterns of the current coastal populations in the North East Atlantic (Chapter 2), whilst the recent demographic and genetic connectivity between the populations occurring in Irish waters are investigated in Chapter 3 (see Fig. 1.5).

After identifying MUs, the abundance and the scale of movements of the 'coastal mobile' population are evaluated in Chapter 4 using a Bayesian mark-recapture approach. Finally, on a smaller spatial scale, site occupancy and habitat use of this population are examined using passive acoustic monitoring (PAM) approaches. The effects of temporal and environmental factors influencing the bottlenose dolphin site occupancy are also evaluated in this chapter.

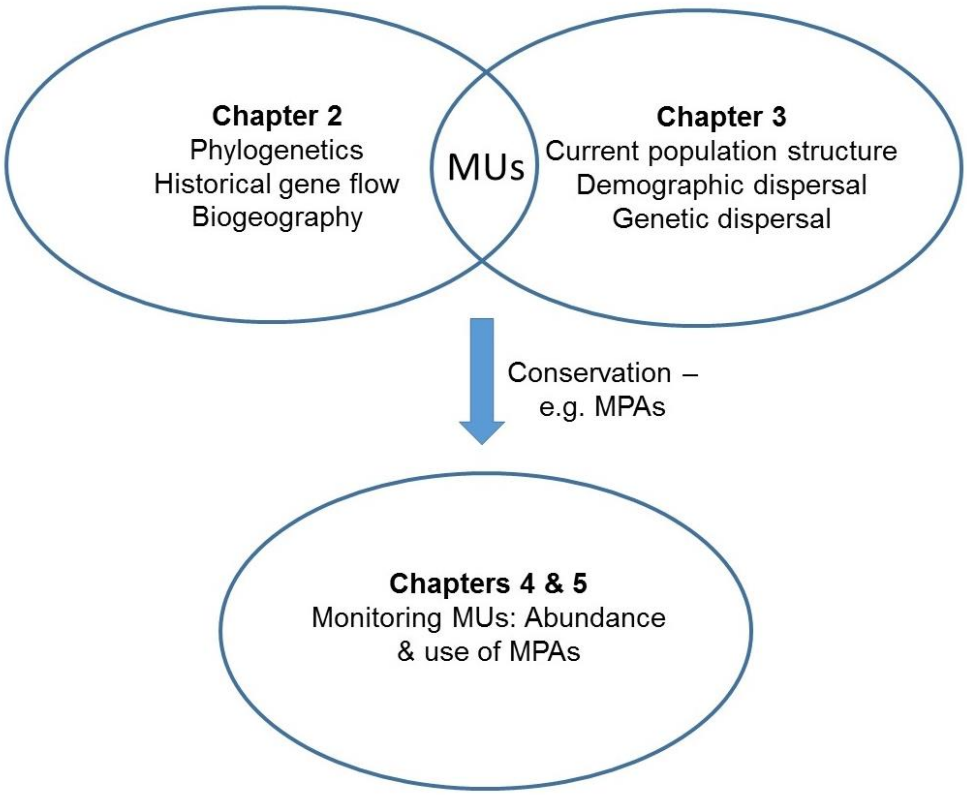

Figure 1.5 A simplified flow chart of the contents of the thesis, which roughly follows the steps in setting a management strategy for the coastal bottlenose dolphins in Irish waters.

All the data chapters in this thesis are written and formatted as journal manuscripts:

Chapter 2: M. Nykänen, K. Kaschner, C. Garilao, V. Biard, A. Brownlow, N. Davison, R. Deaville, W. Dabin, V. Ridoux, F. Gally, P. Gol'din, S. N. Ingram, V. Islas-Villanueva, M. Tange Olsen, E. Rogan, N. Wales, M. Louis and A. D. Foote. *To be submitted*. Modelling the post-glacial colonisation of the Northern extreme of the species range in the bottlenose dolphin *Tursiops truncatus*

Author contributions: **M.N.** and A.D.F. did the laboratory work sequencing the modern samples, A.D.F. trimmed the sequences, V.B., P.G., M.T.O., provided and sequenced the ancient samples, **M.N.** analysed the data, K.K. and C.G. produced the AquaMap suitable habitat maps, M.L., A.B., N.D., W.D., V.R., F.G., E.R. and S.N.I. collected and provided the modern samples. N.W. helped with the lab work, M.L. and V.I-V. provided information on the genotypes. **M.N.** wrote the manuscript. A.D.F., E.R. and S.N.I. supervised the project and commented on the manuscript.

Chapter 3: M. Nykänen, E. Dillane, A. Englund, A. D. Foote, S. N. Ingram, M. Louis, L. Mirimin, M. Oudejans and E. Rogan. *In rewiev*. Quantifying dispersal between marine protected areas by a highly mobile species, the bottlenose dolphin, *Tursiops truncatus*

M.N. and A.D.F. conceptualised the work and the analyses. E.D. and **M.N.** performed laboratory work on the new samples and E.D. and L.M. did the lab work on the existing samples. **M.N.** analysed the genetic data. **M.N**., M.O., A.E. and S.I. collected the photo-identification data, and **M.N.** analysed it. **M.N.**, S.I., E.R., A.F., M.O., and A.E. collected the genetic samples. **M.N.** wrote the manuscript. A.D.F., E.R. and S.N.I. supervised the project and commented on the manuscript.

Chapter 4: M. Nykänen, M. Oudejans, J. Durban, E. Rogan, A. D. Foote and S. N. Ingram. *To be submitted*. Using Bayesian inference with a multi-site mark-recapture model to estimate the abundance of a mobile population of bottlenose dolphins, *Tursiops truncatus*, on the west coast of Ireland

M.N., M.O., E.R., A.D.F. and S.N.I. collected the photo-id data. **M.N.** analysed the data and wrote the manuscript. J.D. provided the code for multi-site abundance estimate. S.N.I., E.R. and A.D.F. supervised the project and commented on the manuscript.

Chapter 5: M. Nykänen, S. N. Ingram, A. D. Foote and E. Rogan. *To be submitted.* Passive acoustic monitoring with C-PODs reveals patterns in site occupancy of bottlenose dolphins, *Tursiops truncatus*, on the west coast of Ireland

M.N. analysed the data and wrote the manuscript. E.R., S.N.I. and A.D.F. supervised the project and commented on the manuscript.

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Chapter 2: Modelling the post-glacial colonisation of the northern extreme of the species range in the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821)

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Abstract

Oscillations in the Earth's temperature and the subsequent retreating and advancing of ice sheets around the polar regions are thought to have played an important role in shaping the distribution and genetic structuring among contemporary populations of plants and animals, especially those occurring at higher latitudes. Following the last glacial period, the retreating ice sheets would have freed suitable habitats for early colonisers to rapidly occupy niches, often leading to the exclusion of other conspecifics, referred to as 'leading edge expansion'. We investigated the post-glacial colonisation of the North East Atlantic (NEA) by bottlenose dolphins, *Tursiops truncatus*, using habitat modelling and genomics. Using the AquaMaps approach, we generated models of suitable habitat for the bottlenose dolphin in the present day and during the Last Glacial Maximum (LGM). According to the model predictions, suitable habitat became available at the northern extreme of this species' range after the LGM. To reconstruct the post-glacial colonisation of the newly available habitat, we generated mitochondrial data from two subfossil samples originating from the Black Sea and archaeologically dated to 1,500 years before present (yBP) and 33 complete mitochondrial genome sequences from modern samples collected in the NEA and assigned to 'coastal' and 'pelagic' ecotypes by previous studies. We then compared these to a published mitogenome dataset. We found little phylogenetic structuring amongst the 'pelagic' samples; in contrast, the 'coastal' NEA samples clustered into two divergent clades. Further, the estimation of the ancestral geographic range of the present day coastal populations at the northern extreme of the species range, inferred that bottlenose dolphins expanded their range northwards from glacial refugial populations that most likely inhabited part of the Mediterranean Sea and lower latitutes of the pelagic Atlantic during the Last Glacial Maximum. Mitochondrial data from the subfossil samples verified a previous biogeographical time-calibration and revealed that coastal bottlenose dolphin populations in the NEA result from a post-glacial radiation by two founder lineages. This pattern of diversity is consistent with the leading edge expansion hypothesis and the associated reduction of genetic diversity. It highlights the legacy of the Late Pleistocene glacial cycles on contemporary genetic structuring.

2.1. Introduction

During the Late Quaternary period (past one million years) the Earth's climate was governed by a series of glacial and interglacial events and temperature fluctuations that occurred in approximately 100,000 year cycles (Shackleton 2000). These glacial cycles are thought to have played an important role in shaping the current distribution and genetic structuring of species and populations. This is more pronounced at high latitudes where the presence of ice sheets and Arctic temperatures during cold stadial periods restricted the available habitat to warmer refugia for many temperate-adapted species (Darwin 1859, pp. 365–382; Hewitt 2000). The distribution of species at high latitudes is thought to have been characterised by cyclical range contractions and expansions throughout the Pleistocene (Hewitt 2000), and that present day populations are relicts of refugia (Hofreiter & Barnes 2010). However, the capacity to adapt to environmental change varies among species and populations through their dispersal ability, genetic diversity and generation time (Stewart *et al.* 2010; Montgomery *et al.* 2014; Younger *et al.* 2016) and has likely affected their present day distribution.

Hewitt (1999, 2000) modelled the post-glacial expansions from glacial refugia for several terrestrial fauna in mainland Europe, identifying Iberia, Italy and the Balkans as the source refugia for the repopulation of more northerly habitat. Some terrestrial fauna in the British Isles appear to have core and Celtic fringe populations, suggesting a two-phase colonisation and partial displacement of the pioneer population to a more restricted Celtic fringe distribution by the second wave (Searle *et al.* 2009; Brace *et al.* 2016). However, comparatively little is known about the post-glacial recolonisation history of Europe's marine fauna. Genetic analyses of carbon-dated sub-fossils indicate that temperate climate adapted species such as grey whales *Eschrichtius robustus* (Lilljeborg, 1861) and North Atlantic right whales *Eubalaena glacialis* (Müller, 1776) replaced cold-adapted species such as bowhead whales *Balaena mysticetus* (Linneaus, 1758) in mid-latitude European waters during the Late Pleistocene-Early Holocene transition (Foote *et al.* 2013; Alter *et al.* 2015). However, the refugial distribution during the Last Glacial Maximum (LGM) of the source populations for these re-colonising temperate marine species is largely unknown.

Understanding the post-glacial colonisation history is a key but often overlooked step to understanding the conservation status of present-day populations. The leading-edge

of range expansions during the warm interglacial periods typically occurred via long-range dispersal events (Hewitt 1999, 2000). Pioneer populations first colonised and then expanded their range to fill emerging geographic and ecological niches leading to the exclusion of secondary waves of colonisers, thereby reducing genetic variability at this leading edge (Hewitt 2000). Much of the empirical support for this hypothesized model of post-glacial recolonisation comes from studies of terrestrial species (Hewitt 2000), but during recent years a growing number of studies have explored the dynamics of postglacial range expansion in aquatic taxa (e.g. deBruyn *et al.* 2009; Foote *et al.* 2013; Catchen *et al.* 2013; Fontaine *et al.*, 2010, 2014). Unlike most terrestrial species, marine species have a relatively low cost of movement (Tucker *et al.* 1975; Williams *et al.* 1992, 1999) and few geographic barriers to dispersal, so the leading edge model may not be realistic for such highly mobile species which are able to move thousands of kilometres within a few weeks (e.g. Gabriele *et al.* 1996; Mate *et al.* 1997) and thus have increased potential to retain ongoing dispersal between core and edge populations.

The circular mitochondrial genome is a widely used marker in phylogenetic analyses due to its non-recombinant properties, fast and clock-like mutation rate and the fact that it is relatively easy to amplify (Duchene *et al.* 2011). However, in the presence of introgression (gene flow by repeated backcrossing of an interspecific hybrid) or incomplete lineage sorting (coalescence time of genes to the same common ancestor and speciation time are different), mitogenomic phylogenies will not necessarily reflect the true species trees (e.g. Amaral *et al*. 2012), and in these cases, nuclear genes or Single Nuclear Polymorphisms (SNPs) are needed to resolve the evolutionary history of the taxonomic groups (Amaral *et al.* 2012; Morin *et al*. 2015). Using complete mitogenomes, rather than single genes or regions such as the control region (CR), can improve the resolution in phylogenetic estimates, especially within species that have undergone rapid radiation (e.g. Yu *et al*. 2007; McGowen *et al*. 2009). However, the use of different mitochondrial regions, such the whole mitogenome or single genes, can lead to contrasting topologies and differing divergence date estimates, probably due to strong purifying selection acting on low variable regions such as the COX2 gene (Duchene *et al*. 2011). Nonetheless, all mitochondrial genes within the Delphinidae family showed clock-like behaviour (Duchene *et al*. 2011), thus they were selected as the marker of choice in this study.

In this study, the post-glacial colonisation of the northern extreme of the range of a top marine predator, the common bottlenose dolphin (*Tursiops truncatus*) was modelled to better understand the dynamics of this process for a highly mobile marine species. The bottlenose dolphin is a cosmopolitan species found throughout the world's tropical and temperate waters (Leatherwood & Reeves 1990), with the northernmost resident population found in the Moray Firth, Scotland at ~58°N. Bottlenose dolphins are found in coastal inshore waters, continental shelf regions and open ocean environments (Wells & Scott 2002). Throughout much of their range, bottlenose dolphins exhibit a hierarchical population structure, with the greatest genetic divergence found between pelagic and coastal populations (Curry & Smith 1998; Hoelzel *et al.* 1998; Natoli *et al.* 2004; Hersh & Duffield 1990; Moura *et al.* 2013; Louis *et al.* 2014a,b; Lowther-Thieleking *et al.* 2015; Allen *et al.* 2016). The broad-scale population structuring between coastal and pelagic ecotypes in the NE Atlantic (Louis *et al.* 2014a) likely reflects colonisation of emerging or available inshore habitats after the Last Glacial Maximum (LGM) from an oceanic source population followed by separation of pelagic and coastal populations who diverged in allopatry (Moura *et al.* 2013; Louis *et al.*, 2014b). These authors suggest that this colonisation of coastal habitats in the NE Atlantic was possibly achieved via a single founder event by a small number of individuals (Louis *et al.* 2014b). In order to understand the climatic, temporal and spatial context of the evolutionary processes that gave rise to the present day diversity of bottlenose dolphins in the North Atlantic, mitogenomic data from contemporary and ancient (sub-fossil) samples was analysed and newly available phylogeographic tools were used that can robustly infer range expansions. Habitat modelling was used to infer the distribution of suitable habitat for bottlenose dolphins during the LGM (~20,000 years before present, yBP). Specifically, the spatial and temporal post-glacial colonisation scenario under which the founding of NE Atlantic coastal populations occurred was reconstructed.

2.2. Materials and Methods

Modern bottlenose dolphin tissue samples from 33 individuals were obtained by biopsy sampling free-ranging dolphins (see Krützen *et al.* 2002) in coastal waters of Ireland and France between 2005 and 2012. Additionally, samples of skin, muscle or kidney tissue were taken from individuals that stranded along the coast of Ireland,

France and the UK between 1991 and 2010 (Fig. 2.1). Samples were selected for sequencing that had been genotyped and assigned as being of 'coastal' or 'non-coastal' origin by previous studies (Islas 2010; Mirimin *et al.* 2011; Louis *et al.* 2014a, see Chapter 3). For consistency with these studies, we refer to individuals not assigned to a coastal population as belonging to a '*pelagic*' ecotype. However, we acknowledge that little is known about the ecology of these individuals, and they may inhabit both neritic and oceanic waters.

To improve node age calibration in our phylogenies, we additionally included material from two ancient sub-fossil samples. Bone material was obtained using the sampling methodology in Morin *et al.* (2006) from two ancient sub-fossil samples (an epiphysis and a vertebrae) originating from a cistern in the Kruze basilica, Chersonesos of Taurica, Crimea. The age of these bone samples is archaeologically estimated as ca. 1,500 yBP based on the excavation depth of the site and by comparison to the rest of the dated objects found in the same layer.

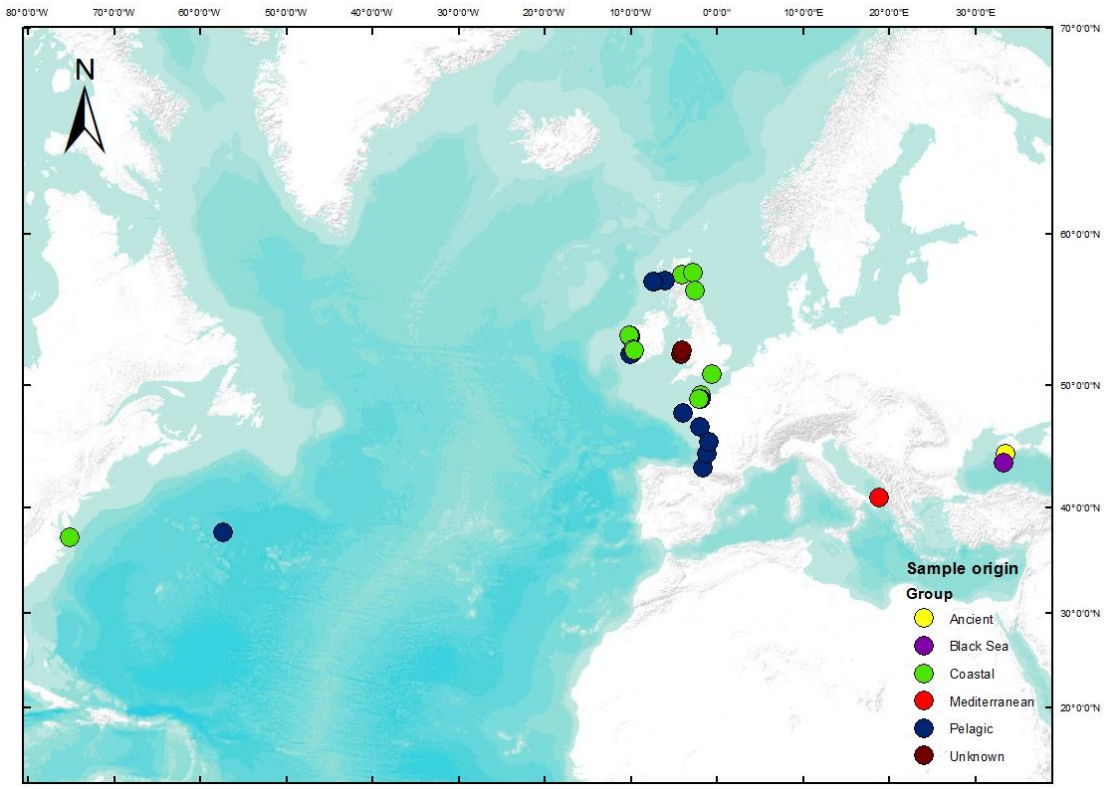

Figure 2.1 Bottlenose dolphin sample locations. Samples have been grouped according to origin based on genotyping in previous studies. Note that the locations for sequences downloaded for GenBank are redrawn from Moura *et al.* (2013). Yellow colour indicates the location of the archeological site where ancient sub-fossil bone samples (n=2) were collected.

DNA extraction, amplification and sequencing

DNA was extracted from modern tissue samples using the Qiagen DNeasy (Qiagen DNeasy, Valencia, CA, USA) kit following the manufacturer's guidelines. DNA yield was quantified using a Qubit (Life Technologies) and ranged between 10 and 300 ng⁄μL for all samples. Modern DNA samples were then sheared to fragments of ~150-200 bp using a Diagenode Bioruptor NGS run with 20 cycles of 30 seconds on, and 30 seconds off.

All pre-amplification laboratory work using the sub-fossil samples was carried out in a designated clean lab, set up specifically for ancient DNA analyses. Blank DNA extractions and PCRs were incorporated to monitor for contamination. No modern whale DNA was present in the same building. Workflow conformed to aDNA protocols, meaning that individuals did not return to the clean lab on the same day following working in post-PCR areas. All post-PCR laboratory work on amplified DNA was conducted in a separate laboratory facility. DNA was extracted and purified from powdered sub-fossil bone using a silica-based method as per Yang *et al.* (1998).

Illumina sequencing libraries were built on the sheared DNA extracts using NEBNext (Ipswich, MA, USA) DNA Sample Prep Master Mix Set 1 following Meyer and Kircher (2010). Libraries were subsequently index amplified for 15 cycles using Phusion High-Fidelity Master Mix (Finnzymes) in 50-μL reactions following the manufacturer guidelines. The libraries were then purified using a MinElute PCR purification kit (Qiagen, Hilden, Germany). Concentrations of amplified libraries were initially checked using a Qubit (Life Technologies) and fragment size distribution was visualized on agarose gel, before a 1/10 diluted aliquot was run on a Agilent Bioanalyser 2100 (Palo Alto, CA, USA) to determine molarity and concentration and facilitate equimolar pooling of index amplified libraries. To generate mitochondrial genome sequences we employed a simple shotgun sequencing method (see Tilak *et al.* 2015). The index amplified libraries were then sequenced in sub-partitions of single channels on an Illumina HiSeq 2500 ultra-high-throughput sequencing platform using single read (SR) 100-bp chemistry.

Conversion of Illumina's *.bcl files to fastq, and demultiplexing were performed using Illumina's CASAVA (version 1.8.2) software allowing for no mismatch in the 6-

nucleotide indices used for barcoding. Sequencing reads within the generated fastq files were processed with Adapter-Removal (Lindgreen 2012), to remove adapter dimers as well as low quality stretches at the 3' ends. Filtered reads were then mapped to a reference *Tursiops* mitogenome sequence (KF570351.1) using BWA version 0.5.9 (Li & Durbin 2009), requiring a mapping quality of Q≥30. The reference mitochondrial sequence was modified as per Morin *et al.* (2015) to improve assembly coverage at the 'ends' of the linearized mitogenome by adding 40-bp from each end to the opposite end (so that reads could map across the artificial break point of the linearized sequence). Clonal reads were collapsed using the rmdup program of the SAMTOOLS (version 0.1.18) suite (Li *et al.* 2009). Ambiguously mapped reads were also filtered out using SAMTOOLS and controlling for XT, XA and X1 tags. Consensus mitogenome sequences were then reconstructed using bam files, which were assembled and visualised in GENEIOUS (Biomatters Ltd.), allowing indels and unique variants to be visually verified in the BAM files.

Phylogenetic and coalescent analyses

Time-dependency in nucleotide substitution rates, where older nodes have lower rate estimates (Ho *et al*. 2011), is a widely acknowledged, albeit debated, issue that can lead to over- or under-estimation of divergence and coalescence times in phylogenetic studies (e.g. Garcia-Moreno 2004; Ho *et al.* 2005; Ho *et al.* 2007; Burridge *et al.* 2008; Ho *et al.* 2011; Subramanian & Lambert 2011; Emerson *et al.* 2015; Ho *et al.* 2015). Time-dependency is thought to occur partly due to incomplete purifying selection, i.e., the removal of transient mutations over longer time periods (Ho *et al*. 2011). Furthermore, the choice of calibration methods and their associated uncertainty to correct for substitution rates has the potential to bias node age estimations (e.g. Ho *et al.* 2005, Rieux *et al.* 2014). A previous study by Moura *et al.* (2013) used a combination of geological and fossil calibrations points in bottlenose dolphin coalescence time estimation and produced convincing ages for younger nodes. However, the authors acknowledged the shortcomings of their study which although estimated more recent node ages consistent with the geological calibration, failed to successfully resolve deeper node ages that were consistent with the fossil calibrations (Moura *et al.* 2013). To overcome these issues a different approach was chosen and only the third codon position sites was used in order to minimize any time-dependency due to incomplete purifying selection, which can lead to overestimation of the

substitution rate over short timescales (Ho *et al.* 2011). Most mutations that occur in third codon positions are silent, or synonymous, coding for the same amino acid (Lagerkvist 1978), and therefore are less likely to be weeded out by purifying selection maintaining a more constant substitution rate over evolutionary time. This approach has been recently used in a study resolving killer whale mitochondrial lineages (Morin *et al.* 2015). However, some mutations at third codons are non-synonymous, in particular transversions (purine ↔ pyrimidine), and putatively short-lived transversions may result in the overestimation of mutation rate. Therefore, an additional approach using a combination of tip calibrations with full mitogenomes sequenced from ancient samples and a fossil calibration point (McGowen *et al.* 2009) applied to a deeper node in the phylogeny was used, since using fossil calibrations of deeper nodes alone can lead to underestimation of substitution rates in more recent nodes (e.g. Rieux *et al.* 2014). To my knowledge, this is the first time that all these techniques have been applied together in population coalescence time estimation.

The assembled mitochondrial genomes, which included 30 modern bottlenose dolphin samples and two ancient subfossil samples sequenced for this study, were aligned using MUSCLE algorithm in software MEGA 6 (Tamura *et al.* 2007) with 44 published *Tursiops truncatus* unique haplotypes from individuals sampled in the North Atlantic and neighbouring marginal seas and a rough-toothed dolphin *Steno bredanensis* sequence (used as an outgroup) available in GenBank (see Appendix 2.1 for sequence accession numbers). A topology tree in MRBAYES (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) was then built with these sequences after the initial model selection for the best substitution scheme in JMODELTEST 2 (Darriba *et al.* 2012, Posada 2008), and the resulting consensus tree was inspected to find the two most divergent North Atlantic bottlenose dolphin haplotypes (WNAC13 and BSEA5).

The thirteen protein coding genes of the mitochondrial genome from these two most divergent North Atlantic *T. truncatus* haplotypes were aligned with the gene sequences of an additional 19 delphinids (Appendix 2.1) downloaded from GenBank. The greedy search in PARTITIONFINDER (v1.1.0) (Lanfear *et al.* 2012) was used to find the best partitioning scheme for each gene and codon separately, and a time-calibrated phylogenetic tree was built in BEAST 2.3.2 (Drummond *et al.* 2012) with five different data partitions for nucleotide substitution models (Appendix 2.2) in order to find the

time to most recent common ancestor (TMRCA) between the two most divergent North Atlantic bottlenose dolphins. The split between Monodontidae and Delphinidae (average 10.08 MyBP, SD = 1.413 MyBP, McGowen *et al.* 2009) was used to calibrate the root of the tree and a Yule prior for the branching rate. Both uncorrelated lognormal relaxed clock and strict clock models with 20,000,000 Markov Chain Monte Carlo (MCMC) steps, 10% burn-in, and a sampling frequency of 2,000, were examined. Each model was run twice and the convergence of chains and the Effective Sample Size (ESS) values relating to the model parameters were checked in TRACER 1.6 (Rambaut *et al.* 2012). After verifying convergence, LOGCOMBINER and TREEANNOTATOR (Drummond *et al.* 2012) were used to combine and summarize the trees. Model selection between the two clock models was done by inspecting the ucldStdev parameter in the relaxed clock model; a standard deviation of close to zero (<0.1) in this parameter indicates no variation in the substitution rates across branches and a better fit of the strict clock model (Drummond & Boukaert 2015).

The best nucleotide partitioning scheme for the coalescent analysis of North Atlantic bottlenose dolphins was determined for the thirteen protein coding gene regions of the 74 modern and two ancient bottlenose dolphin mitochondrial genomes using PARTITIONFINDER (v1.1.0) (Appendix 2.2). Only the third codon positions of the protein coding genes were used following Morin *et al.* (2015), in order to minimize the effect of incomplete purifying selection, which can potentially lead to overestimation of the substitution rate on short timescales (Ho *et al.* 2011). A constraint on the root age obtained in the previous step (split of the two most divergent North Atlantic bottlenose dolphin haplotypes, mean 0.8 My, SD = 0.14) was used and tip calibrations with the two ancient sequences dated to 1,500 yBP. Three different Bayesian coalescent models were tested with a strict molecular clock in BEAST 2.3.2 (Drummond *et al.* 2012); models with a constant and an exponential population size and a Bayesian Skyline model that allows changes in the (female) effective population size through time. Each of the models were run twice using 40,000,000 MCMC steps, 10% burn-in and a sampling frequency of 10,000. The convergence of chains and model performance was inspected in TRACER 1.6 (Rambaut *et al.* 2012). The final model selection was done by calculating Bayes factors obtained by Path Sampling method (Baele *et al.* 2013) also run in BEAST 2.3.2 with 150 steps. After the best model was selected, the derived mean node ages (i.e. times to MRCA, see Fig. 2.4) were

compared to the node ages reported in Moura *et al.* (2013) by plotting them and visually comparing the fit of the line to linear regression.

Whether there was a significant population bottleneck or expansion indicative of contraction or release of habitat after the LGM was tested by calculating nucleotide diversity (π), Tajima's *D* (Tajima 1989) and Fu's *F* (1997) statistics with DNASP 5.10.1 (Rozas *et al.* 2009) for the protein coding gene regions for all the samples combined. These statistics were also used for samples grouped as 'coastal north' and putative 'pelagic' ecotypes based on nuclear microsatellites by previous studies (Islas 2010; Mirimin *et al.* 2011; Louis *et al.* 2014a) and their placement in the phylogeny is presented in Fig. 2.4.

Phylogeographic methods

Operational Taxonomic Units (OTUs) were selected from the time-calibrated coalescent North Atlantic *T. truncatus* tree (Fig. 2.4) as one representative sample from each clade/haplogroup with a posterior probability for the associated node of ≥ 0.80 leading to 14 OTUs. The package BIOGEOBEARS (Matzke 2013, 2014) in R (R Core Team 2016) was used to compare different phylogeographic scenarios. The benefit of this package is that the founder-event speciation is also considered in the models along with traditional dispersal-extinction-cladogenesis processes (Matzke 2013, 2014), and that the model selection can be done with nested and non-nested models using AIC implemented in the R-code. Six different geographic areas were defined based on the sampling locations (and the populations defined in previous studies) of the tips of the tree; North West Atlantic coastal (C), North Atlantic pelagic (P), Ireland and British Isles coastal (I), France coastal (F), Mediterranean Sea (M) and the Black Sea (B), and altogether six different models were tested, with and without founder event speciation (see Matzke 2013, 2014) to reconstruct the likely biogeographic history of bottlenose dolphins across the North Atlantic. Each model, namely DEC, DIVALIKE and BAYAREALIKE, allows for a different combination of biogeographical scenarios, such as dispersal (i.e. range expansion), vicariance (i.e. range splitting) and extinction (i.e. range contraction) and estimates the rate of these events along the phylogeny branches (Matzke 2013, 2014). All of these models include terms describing dispersal, extinction and some form of sympatry (i.e. daughter lineages inheriting the ancestral ranges), but unlike DEC and DIVALIKE models, the BAYAREALIKE model

assumes no vicariance occurring in cladogenesis, but that ancestral ranges are passed on to daughter lineages as they are (see Matzke 2013). A maximum of three areas was allowed to be inferred as the ancestral distribution at each node, meaning that the tips of each clade would not occur in more than three areas, which is a reasonable assumption considering the current observed population structure of the bottlenose dolphins in the North Atlantic (e.g. Hoelzel *et al.* 1998, Louis *et al.* 2014a, Mirimin *et al.* 2011, Natoli *et al.* 2005, Gaspari *et al.* 2015). Time-stratified analyses were run using modelled historical Sea Surface Temperatures (SSTs) and geological events to infer areas suitable for colonisation by bottlenose dolphins at different time points based on their modern day distribution and the AquaMaps models for suitable habitat. Specifically, the coastal areas of the NW Atlantic and the pelagic waters of lower latitudes were defined as suitable for bottlenose dolphins during the entire time period considered (ca. 800,000 years), and the Mediterranean Sea suitable for colonisation since the onset of the Eemian interglacial ca. 130,000 yBP (e.g. Martrat *et al.* 2004). The northern parts of the NE Atlantic were considered suitable for colonisation since 10,000 yBP when the mean annual SST remained consistently at or above 10-11°C (Feng & Ogelsby 2009), a similar average annual temperature to the Moray Firth, Scotland (http://www.metoffice.gov.uk), that holds a current resident population of bottlenose dolphins. The Black Sea remained closed from the Mediterranean Sea between ~10 MyBP and ~10 Kya (e.g. Kerey *et al.* 2004), but the earliest Mediterranean marine fossil species indicative of marine, rather than brackish, conditions have been dated to 7,200–5,000 yBP during the Kalamitian transgression (Yanko-Hombach *et al.* 2002) so the Black Sea was considered to be inaccessible for bottlenose dolphin colonisation until this time point.

Models of suitable habitat

AquaMaps modelling approach (Ready *et al.* 2010; Kaschner *et al.* 2011; www.aquamaps.org) was used to predict the distribution of suitable habitat for common bottlenose dolphins (*T. truncatus*) during the present, forecast for the year 2100 under the Institut Pierre Simon Laplace (IPSL) climate scenario SRES A2, and hindcast for the LGM (~20,000 yBP). AquaMaps is a bioclimatic model that combines existing locations of occurrence from visual observation data with available expert knowledge on species preference and tolerance to different environmental parameters and ultimately generates large-scale predictions of the probability of occurrence for

different marine species. This way the preferred habitat of a species can be estimated based on a predefined set of environmental parameters including water depth, sea surface temperature (SST), salinity, primary production, sea ice concentration and proximity to land, which is subsequently projected into geographic space in a global grid of 0.5° latitude by 0.5° longitude cells. For the purpose of this study, a slightly modified version of the AquaMaps default model was used (which is available on www.aquamaps.org); specifically, primary production was excluded from the model, as there are no available data for this parameter for the Pleistocene. Predictions of relative probability of bottlenose dolphin occurrence were projected into geographic space based on local conditions using environmental data for different time periods and assuming no changes in habitat use over time. Current environmental conditions were assumed to be representative of the entire Holocene as the variability in conditions during this time period has been small compared to the differences between glacial cycles (Folland *et al.* 2001). Current distribution was based on the compiled standard AquaMaps environmental data, as described by the meta-data available at aquamaps.org/download/main.php. AquaMaps has been previously used to hindcast suitable habitat predictions for bowhead whales, grey whales and killer whales during the LGM (Foote *et al.* 2013; Alter *et al.* 2015; Morin *et al.* 2015) by using mean annual environmental conditions during the LGM based on the GLAMAP project data set (Schäfer-Neth & Paul 2003; see Foote *et al.* 2013). The approach used in these previous studies (Foote *et al.* 2013; Alter *et al.* 2015; Morin *et al.* 2015) was followed and distribution maps were constructed of core suitable habitat for bottlenose dolphins during the present time and hindcasted for the LGM.

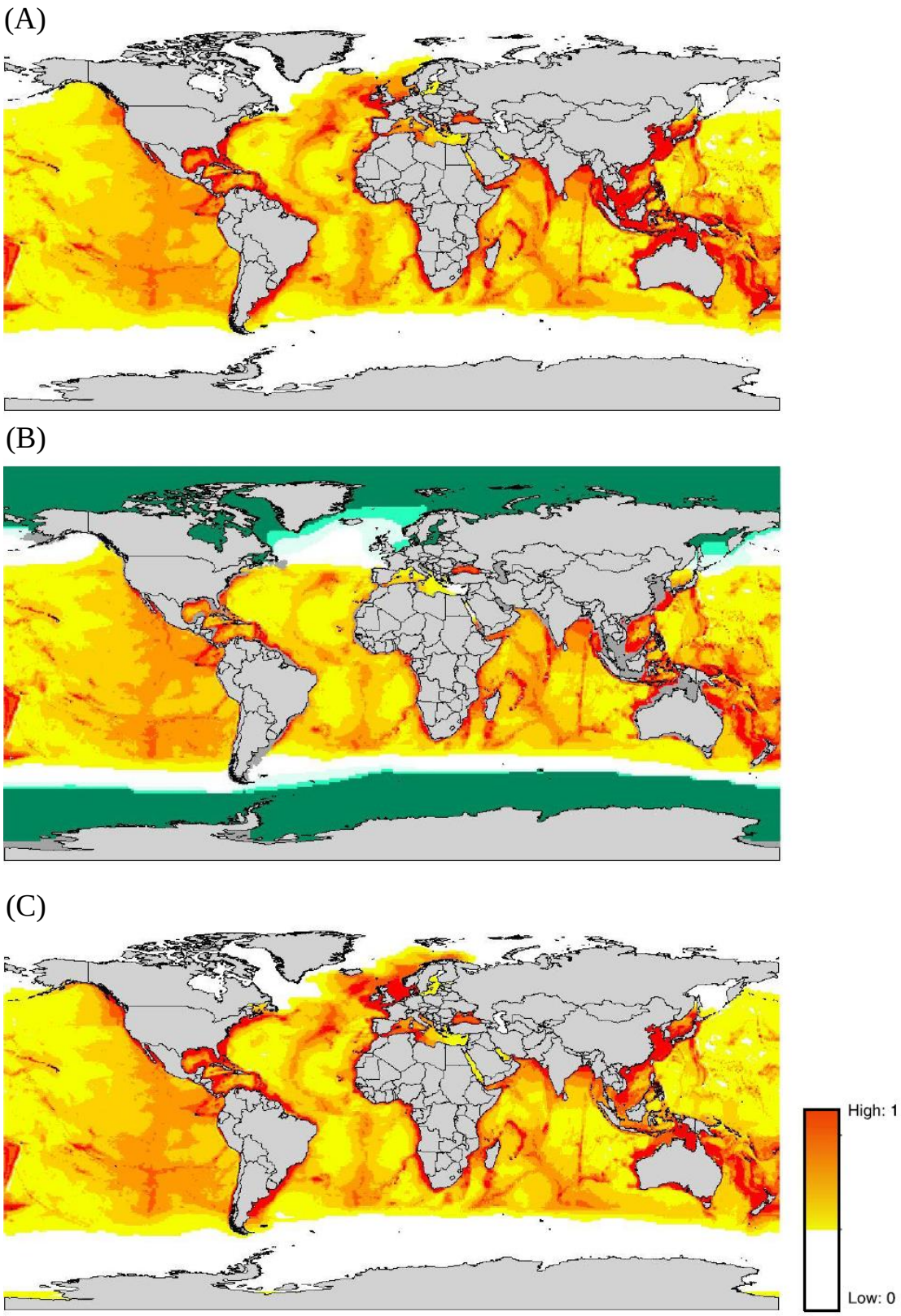

Figure 2.2 AquaMaps suitable habitat map for bottlenose dolphins for the (a) present day, (b) the last glacial maximum (LGM), and (c) the year 2100. White to red colours represent least to most suitable habitat, respectively, based on the AquaMaps habitat model (see Materials and methods). Land is shown in light grey. Dark green colour in figure (b) represents areas with permanent ice sheet and light green colour areas with >50% sea ice concentration.

2.3. Results

A total of 292×10^6 sequencing reads were generated from the modern samples; >1 million reads per individual for 28 of the 33 individuals included on the sequencing lane. Following QC filtering, removal of duplicate reads, mapping and only including nucleotide positions with a read depth of ≥3× coverage, complete mitochondrial genomes were assembled for 30 individuals with a mean sequence coverage ranging from 10 to >100×, there were insufficient data for reconstruction of complete mitochondrial genomes for three individuals, which were therefore excluded from further analyses. The control region sequence for each individual was compared with those generated by Sanger sequencing by previously published studies (Islas 2010; Mirimin *et al.* 2011; Louis *et al.* 2014a), in some cases the same individual had been previously sequenced independently by all three studies. In all cases our high-throughput sequencing generated data agreed 100% with the Sanger sequence data. Altogether 64 unique modern sequences were identified, including 42 previously identified haplotypes from Moura *et al.* (2013). 20 new haplotypes from the modern samples sequenced for this study were also identified (Appendix 2.1), some of which are already deposited in GenBank (KT601188-KT601207). Additionally, the two ancient samples from the Black Sea, which were sequenced across a separate sequencing lane to the modern samples, yielded whole mitochondrial genome sequences with mean coverage of 26× and 28× each. These mitogenome sequences were also identified as new haplotypes, BSEA11 and BSEA12 (Appendix 2.1), and they will be submitted to GenBank.

Calibration of root node age

The low standard deviation (0.087) of the clock rate in the model with the uncorrelated log-normal relaxed clock indicated low variation in substitution rates among delphinid lineages (branches) and was considered a better fit of the strict clock model. This was also found by Duchene *et al.* (2011) for delphinids. Average substitution rate for the strict clock model was estimated as 0.0112 substitutions/site/My (SD = 0.0019). The average time to the most recent common ancestor (TMRCA) of the two most divergent North Atlantic bottlenose dolphins was

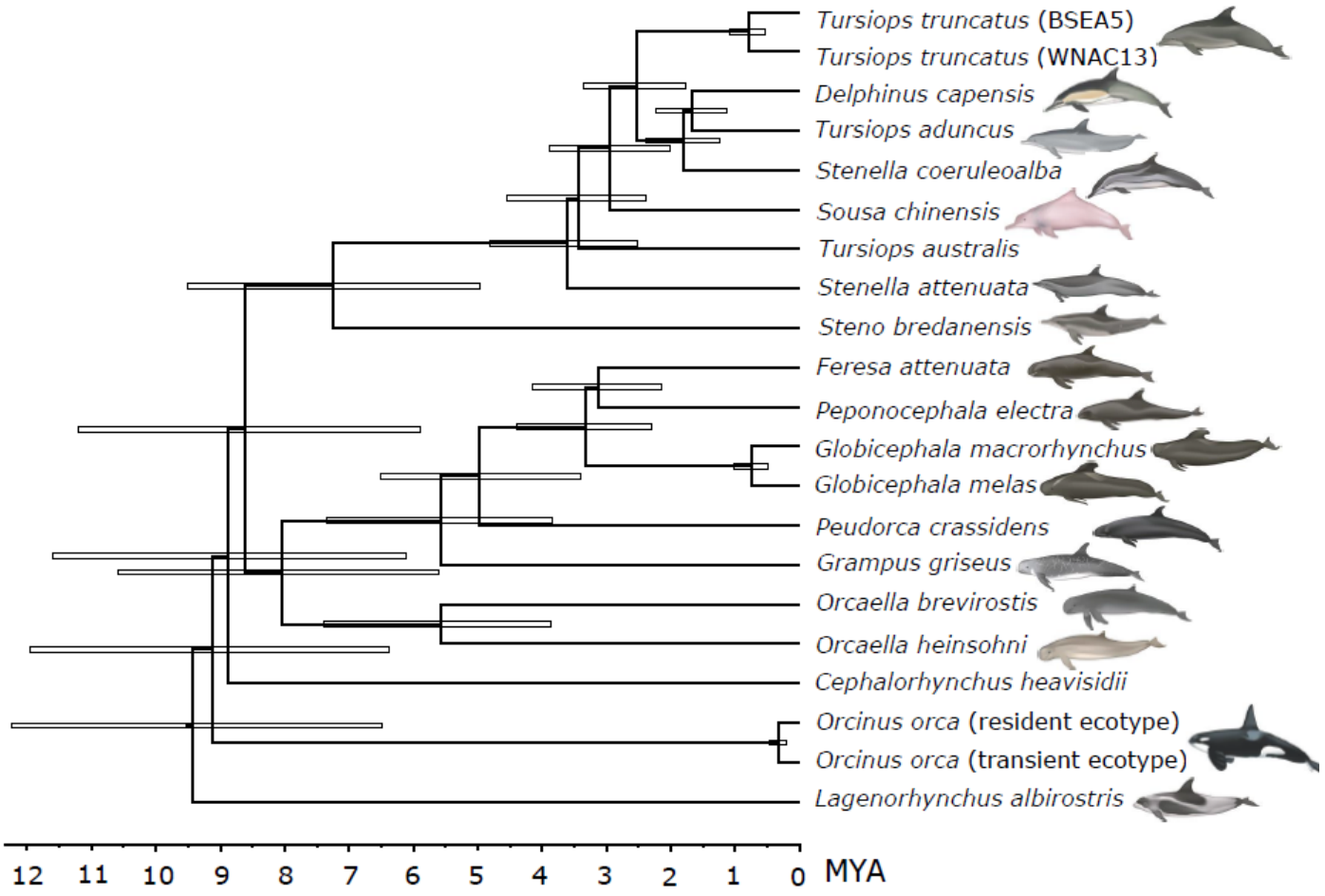

Figure 2.3 Time-calibrated delphinid phylogeny. The time scale is given in millions of years, and the numbers and bars represent mean node age with 95% HPDI, respectively. Samples coded as BSEA5 and WNAC13 are the two most divergent NE Atlantic bottlenose dolphin samples analysed in this study.

estimated as 0.80 MyBP (95% HPDI: 0.53–1.1 MyBP, SD = 0.14, Fig. 2.3). The average TMRCA of the two killer whales included in the analysis (samples from transient and resident north Pacific ecotypes) was estimated as 0.33 MyBP (95% HPDI: 0.19–0.46 MyBP); this is very similar to a previously reported estimate for divergence of killer whales of 0.36 MyBP (95% HPDI: 0.22– 0.53 MyBP; Morin *et al.* 2015).

Time-calibrated phylogeny of North Atlantic bottlenose dolphins

The ESS values for the parameters in the coalescent models were generally >200 (most of them >1000) indicating no sign of autocorrelation between samples and a good convergence of chains (Table 1). The only exception to this was the 2nd run of the Bayesian Skyline model, where some of the likelihood parameters for the individual gene topologies had low (<200) ESS values. Inspection of the marginal probability plots of these parameters revealed a weak bimodal distribution, which was likely to be a result of some the individual gene topologies not fully supporting the consensus topology tree created by combining all of the gene trees (Duchene *et al.* 2011; Drummond & Bouckaert 2015). However, this was considered to have negligible effect due to the small area represented by the secondary peak in the marginal probability distribution.

The marginal posterior distribution of the growth rate variable in the coalescent model with exponential population size included zero; therefore, the model with constant population size was a better fit to the data (Drummond & Bouckaert 2015). The summary consensus tree made using this model (Fig. 2.4) indicates that one of the clades consisting of coastal samples collected in Western Ireland, West of Scotland and North of France (Brittany) coalesced to a common ancestor with a clade consisting of Mediterranean and Black Sea samples ca. 45 kyBP (95% HDPI: 18–78 kyBP), while another clade including coastal samples from Western Ireland, East Scotland, Wales and Northern France coalesced to a clade that includes both pelagic and French coastal samples around 55 kyBP (95% HDPI: 18–100 kyBP) and to a clade with Mediterranean samples shortly before this, approximately 58 kyBP (95% HDPI: 24–95 kyBP). The inspection of the correlation of the estimated times to

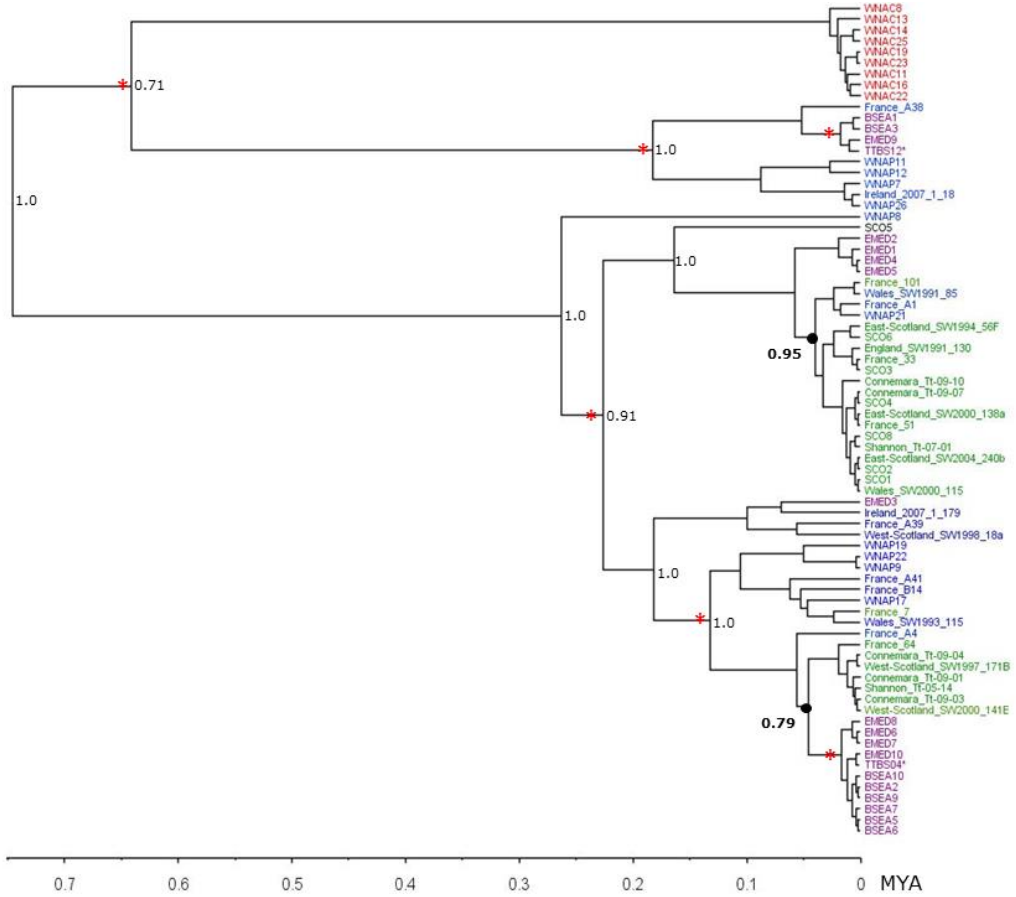

Figure 2.4 Time-calibrated North Atlantic *Tursiops truncatus* tree created using the best fitting BEAST model, Bayesian coalescent with constant population size, with a constraint on the root of the tree and two tip calibrations (indicated with an asterisk after the sample name). Colours denote origin of the sample; red – NWA coastal, blue – pelagic, green – NEA coastal, purple – Mediterranean/Black Sea. Red asterisks mark the nodes used in Fig. 2.5. Numbers denote the bootstrapped node posterior probabilities.

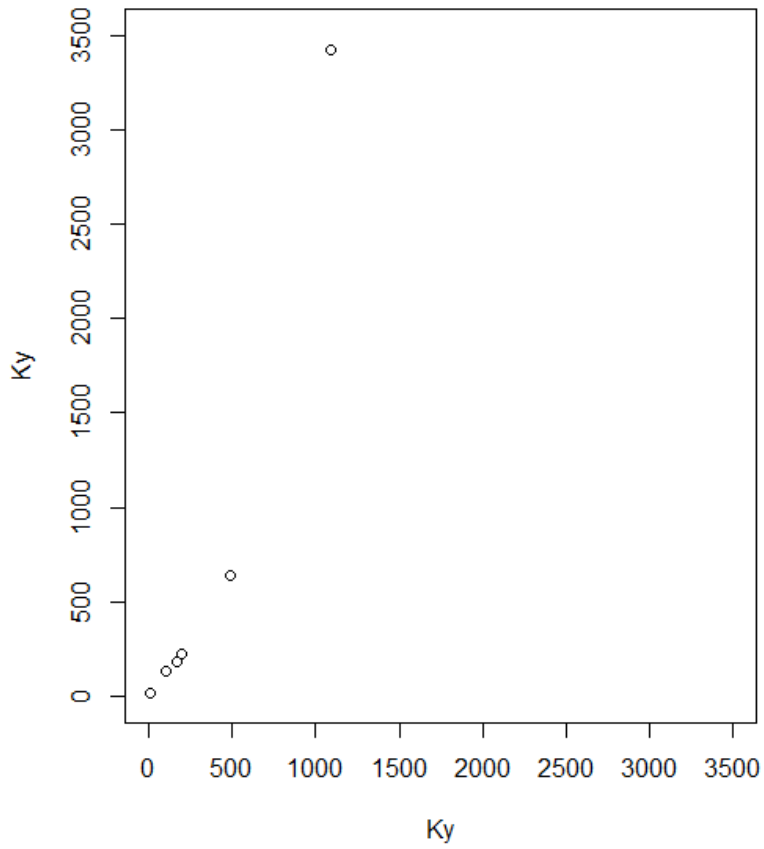

Figure 2.5 Correlation of coalescence times estimated in this study (y-axis) and in a study by Moura *et al.* (2013) (x-axis) represented by circles.

the MRCA (Fig. 2.5) revealed that whilst the age of the younger nodes reported by Moura *et al.* (2013) corresponded well with the age of the younger nodes derived in this study, the estimates for deeper nodes are much older than those in Moura *et al.* (2013).

The average substitution rate for the third codon positions across all branches for the bottlenose dolphins in the North Atlantic was estimated as 0.017 substitutions/site/My (95% HPDI: 0.010–0.026). This estimate corresponds well with previously estimated rates of 0.024 (95% HPDI: 0.022–0.026) for third codon positions for cetaceans (Ho & Lanfear 2010) and 0.031 substitutions/site/My and 0.021 substitutions/site/My for bottlenose dolphin mitogenome-wide rates derived using IMa and BEAST software, respectively (Moura *et al.* 2013).

The coalescent model with constant population size (CP) (Fig. 2.4) was also a better fit to the data compared to the Bayesian Skyline (BS) model according to the path sampling model comparison method (Baele *et al.* 2013), but the difference in the marginal likelihoods between these two models was negligible (CP: -6798.2, BS: -6798.8). In addition, both models gave almost identical estimates for node MRCA times and clock rates; therefore, historical changes in female effective population size was reconstructed as a Bayesian Skyline plot, and a reduction and a subsequent increase in population size at ~25,000 yBP and ~10,000 yBP, respectively, was identified (Fig. 2.6). Further, the significantly positive Tajima's *D* value calculated for the samples from the 'Coastal North' population indicated that this population may have suffered from a recent bottleneck (Table 2.1), also supported by the reduced number of segregating sites (k) and low nucleotide diversity (π) associated with this group. All the other groups, and when all the samples were combined, had non-significant indicator values reflecting relatively constant recent population sizes or no selection affecting on the genetic loci.

Models for suitable habitat and phylogeography

The AquaMaps model estimated that all core suitable habitat for bottlenose dolphins was distributed South of the Iberian Peninsula (~40° latitude) in the NE Atlantic during the LGM. Core suitable habitat in the North Atlantic during the LGM was hindcast to cover the areas of the coastal Western Atlantic (up to approximately the latitude of North-Carolina), along the mid-Atlantic ridge and also part of the Mediterranean Sea (around the Alboran Sea) (Fig. 2.2b). Note that AquaMaps hindcast suitable habitat also in the Black Sea, however, this remained closed and thus inaccessible for the dolphins until after 10,000 yBP (Kerey *et al.* 2014).

Table 2.1 Indicators of neutral vs. non-neutral sequence evolution used to infer recent NE Atlantic bottlenose dolphin population expansion/contraction.

Population	k	Tajima's *D*	π
All samples (n=74)	354	-0.45	0.0055
Coastal North (n=21)	50	2.05*	0.0019
Pelagic (n=22)	239	-0.38	0.0052

k = number of segregating sites, i.e. polymorphisms
π = nucleotide diversity
*$P < 0.05$

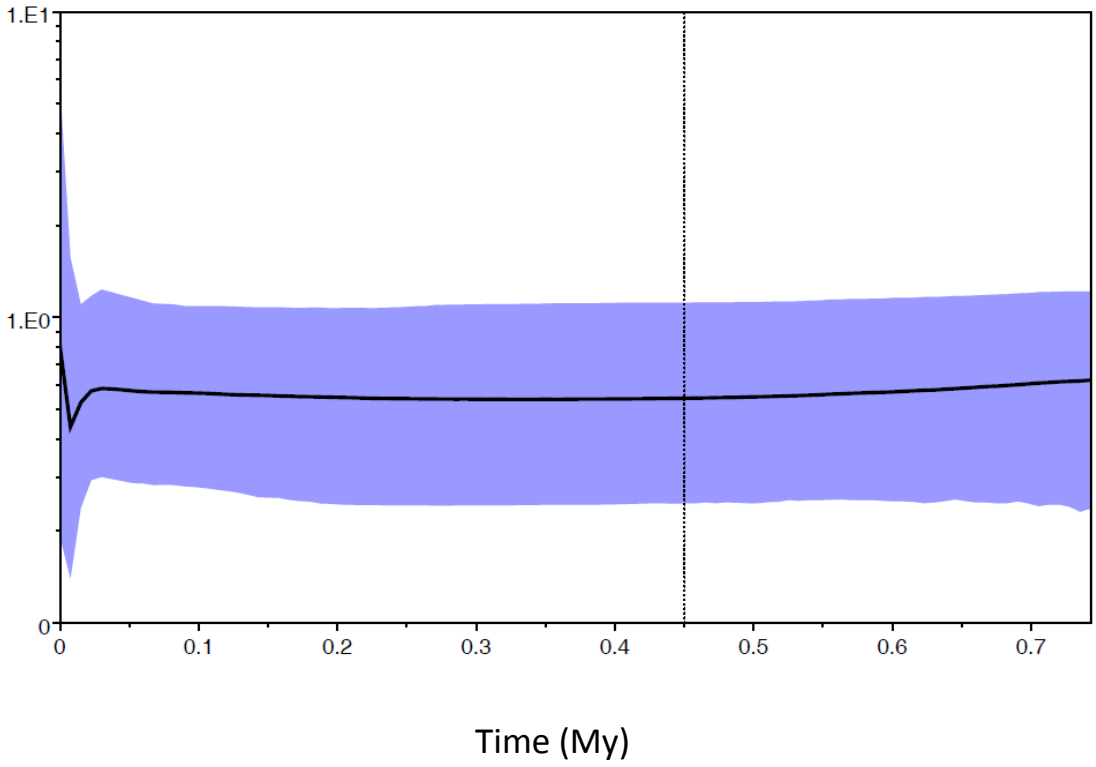

Figure 2.6 Bayesian Skyline plot representing changes in the median female effective population size (*Ne*) of the North Atlantic bottlenose dolphins during the past 0.7 million years (My). Note that y-axis is scale is relative, with 1.E0 = 1 and 1.E1 = 10.

The best phylogeographic model for inferring ancestral ranges of North Atlantic bottlenose dolphins was the BAYAREALIKE+J model with founder-event speciation (AIC = 120.8). This model had significantly better fit compared to the BAYAREALIKE model without founder event (AIC = 144.6, $P < 0.001$) indicating that founder event (or jump dispersal) was an important process in the dispersal of bottlenose dolphins in the North Atlantic. Conversely, cladogenetic events (vicariance and subset speciation, see Methods section and Matzke 2013) were inferred as not being important processes in establishing the present day phylogeography of maternal lineages. The ancestors of North Atlantic bottlenose dolphins were most likely occupying the coastal waters of the NW Atlantic and Atlantic oceanic waters (Fig. 2.7). The NE Atlantic coastal clade consisting of haplotypes from West of Ireland and Western Scotland likely descended from ancestors occupying the Mediterranean Sea who then dispersed to pelagic waters before colonizing the northern coastal parts of the NE Atlantic (Figs. 2.4 and 2.7). Similarly, founder events via the Mediterranean are likely to have played a role in the colonisation of coastal areas of East Scotland, Northern France and Wales (Figs. 2.4 and 2.7).

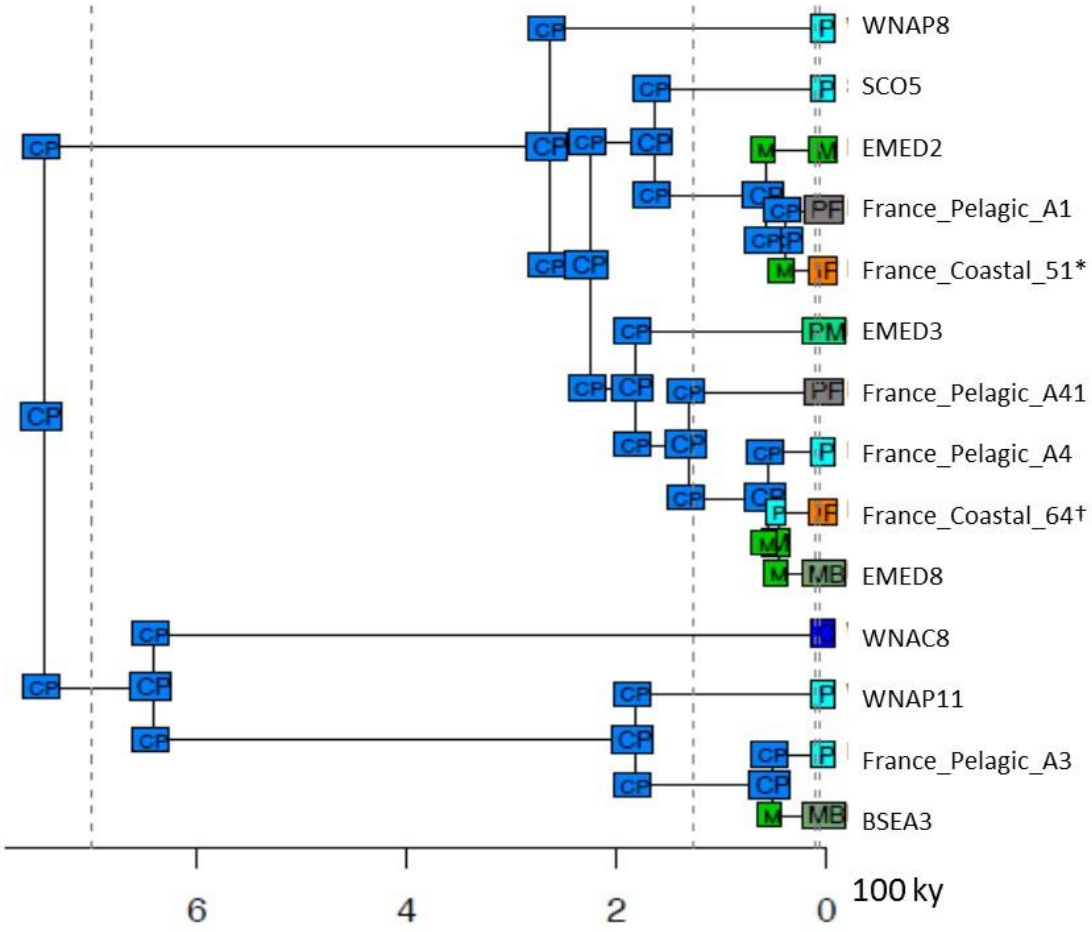

Figure 2.7 The most probable ancestral ranges of *Tursiops truncatus* in the North Atlantic estimated using the best fitting BIOGEOBEARS model BAYAREALIKE+J from the phylogeny represented in Fig. 2.4. The corner positions represent the geographic range immediately after a cladogenesis event. Coloured boxes represent ranges with letters C = NWA coastal, P = Pelagic, M = Mediterranean, B = Black Sea, I = Ireland & UK coastal, F = France coastal. Tips (sample names) have been collapsed from Fig. 2.4 as one representative sample for each clade/lineage. The lineage marked with an asterisk and mainly consisting of samples from East Scotland, Northern France and Wales likely descended from ancestors occupying the Mediterranean Sea and before that the coastal areas of NWA and the pelagic via founder events. The lineage marked with a symbol † and represented by samples from West of Ireland and Western Scotland likely descended from ancestors occupying the pelagic waters and before that, the Mediterranean Sea, also by jump dispersal.

Table 2.2 Mean parameter and ESS values for Bayesian phylogenetic and coalescent models run in BEAST.

Delphinids

Model: Yule with strict clock

	Posterior	Likelihood	Prior	Tree height	*T. truncatus* TMRCA	Clock rate	ucldStdev
Mean	-43377.55	-43254.03	-123.52	9.43	0.7996	0.0112	NA
ESS	3079	2677	2595	17259	16508	16783	NA

Model: Yule with log-normal relaxed clock

	Posterior	Likelihood	Prior	Tree height	*T. truncatus* TMRCA	Clock rate	ucldStdev
Mean	-43350.39	-43221.89	-128.50	9.41	0.7640	0.0113	0.0873
ESS	2694	1908	1448	9001	7995	8297	4233

Tursiops truncatus

Model: Constant population size

	Posterior	Likelihood	Prior	Tree height	Clock rate (strict clock)
Mean	-6575.85	-6517.50	-58.34	0.75	0.0172
ESS	5774	5648	7235	8452	8306

Model: Bayesian Skyline

	Posterior	Likelihood	Prior	Tree height	Clock rate (strict clock)
Mean	-6571.64	-6517.90	-53.74	0.74	0.0176
ESS	3475	3836	3530	8141	7399

Model: Exponential population size

	Posterior	Likelihood	Prior	Tree height	Clock rate (strict clock)
Mean	-6578.58	-6517.76	-60.83	0.72	0.0181
ESS	840	791	563	880	627

2.4. Discussion

Sequencing complete mitochondrial genomes from multiple coastal populations and from a pelagic population of bottlenose dolphins in the Northeast Atlantic led to the discovery of several distinct lineages/clades of mostly either pelagic and coastal individuals. Further, the estimation of the ancestral geographic range of the present day coastal populations at the northern extreme of the species range inferred that bottlenose dolphins expanded their range northwards from glacial refugial populations that most likely inhabited part of the Mediterranean Sea and lower latitude pelagic waters during the Last Glacial Maximum. The signature of expansion suggests this colonisation may have been from a small founder group that comprised as few as two maternal lineages.

Pleistocene climatic oscillations are thought to have played a major role in shaping species distribution and divergence and in promoting speciation (Avise & Walker 1998). According to the time calibrated tree (Fig. 2.4), the splitting of the West North Atlantic coastal clade from other clades at around 650,000 yBP coincides with the end of a cooler period during the middle Pleistocene when the SST started to gradually rise again up to ~10°C in the northern hemisphere (Clark *et al.* 2006). Another split between a pelagic clade and clades consisting mostly of NE Atlantic coastal and Mediterranean/Black Sea haplotypes coincides with the onset of the warmer Eemian interglacial ~130,000 yBP, when temperatures rose nearly by 10°C within a few thousand years (Rasmussen *et al.* 2003). It thus appears that these cladogenesis events are correlated with periods of temperature changes, with warmer temperatures leading to an increase in sea-level in coastal areas and the subsequent release of available habitat.

Temperature changes after the LGM have likely played a role also in the colonisation of the northern parts of the NE Atlantic. Nearly all the coastal haplotypes in the NE Atlantic (clades indicated in green colour in Fig. 2.4) have radiated from just two ancestral lineages; thus the colonisation of the coastal NE Atlantic would have occurred via at least two founding females. The TMRCA of both of the coastal NE Atlantic clades date after the onset of deglaciation: at ~19,000 yBP and ~15,000 yBP, respectively, indicative of demographic expansion once the coastal habitat was colonised. The recent nature of this post-glacial population expansion is further

supported by the significantly positive Tajima's *D*, reduced number of segregating sites and diversity, and the rapid population expansion evident in the Bayesian Skyline plot at ca. 10,000 yBP, which coincides with the temperatures becoming warmer in the northern parts of the NE Atlantic causing a rise in sea levels and the likely increase of suitable habitat. The phylogeography model inferred that the ancestors of both clades dominated by NE Atlantic coastal bottlenose dolphins were probably established in the Mediterranean before colonizing the coastal areas of the NE Atlantic. These may represent two concurrent, but independent colonisation events, or one founding event by a group with at least two divergent maternal haplotypes. The results from a recent study by Louis *et al.* (2014a), however, suggests one founding event based on evidence from nuclear microsatellites.

Post-glacial TMRCA of the Mediterranean/Black Sea clades in this study are concordant with the estimated timing of spatial and demographic range expansion in Gaspari *et al.* (2015) suggesting that rapid radiation and expansion in the Mediterranean Sea likely occurred after the LGM. However, based on the TMRCA of the NE Atlantic coastal clades and their respective Mediterranean/Black Sea sister clades, it seems that the initial colonisation of the Mediterranean Sea may have occurred much earlier, i.e. around 45-55,000 yBP by two lineages (Figs. 2.4 and 2.7). This is supported by the hindcast model for core suitable habitat distribution during the LGM (Fig. 2.2b), which inferred part of the Mediterranean Sea as one the few areas of core suitable habitat in the NE Atlantic along with coastal areas of the NW Atlantic and the lower latitudes of the pelagic waters. Furthermore, even if northern parts of the Mediterranean Sea (e.g. Tyrrhenian Sea) were too cold or otherwise unsuitable for the bottlenose dolphins during LGM (as argued by Gaspari *et al.* 2015), a refugial population may have existed in the areas around the warmer Alboran Sea, with models of SST showing consistently warmer annual temperatures above 10°C for this area throughout the last ice age (Cacho *et al.* 2001). Expansion towards the northern (and inner) parts of the Mediterranean, however, likely occurred after the LGM, as supported by the post-glacial TMRCA of the Mediterranean/Black Sea clades in this study and also in Moura *et al.* (2013), as well as the timing of expansion estimated by Gaspari *et al.* (2015). Similar refugia at lower latitudes have been documented for example for Atlantic salmon, *Salmo salar* (Consuegra *et al.* 2002; Finnegan *et al.* 2013) and European eel, *Anguilla anguilla* (Kettle *et al.* 2008). Even during periods of

very rapid cooling and depletion in salinity during Younger Dryas (12,900–11,700 yBP, Bakke *et al.* 2009), bottlenose dolphins may have responded by shifting their range south or eastwards to more suitable areas. Migration and shifts in distribution have been suggested as a way for mobile species to cope with changes or loss of habitat by several authors (e.g. see review by Hughes 2000; DeBruyn *et al.* 2009).

Hewitt (1999, 2000) hypothesized that early colonisation to high latitudes during warm interglacial periods would occur via long-range dispersal events, that these early colonisers would expand to fill areas and occupy niches before others arrived and the population would grow exponentially, and that these processes would lead to reduced genetic variability at this leading edge. The first hypothesis is generally applicable to cetaceans, such as the bottlenose dolphin, due to their ability for long range movements on relatively short time-scales (e.g. Forcada *et al.* 2009; O'Brien *et al.* 2012; Robinson *et al.* 2010). The colonisation and expansion to fill the available coastal habitat around the British Isles by two mitochondrial lineages, in which there is very little evidence for introgression by pelagic lineages as shown by this study, and Louis *et al.* (2014a), is also consistent with the model of a leading edge expansion (Hewitt 2000). Serial founder events leading to sequential loss of genetic diversity have been linked to the phylogeographic history in a number of species, including modern humans (*Homo sapiens*) during the migration out of Africa (e.g. Ramachandran *et al.* 2005; DeGiorgio *et al.* 2009; Deshpande *et al.* 2009, Henn *et al.* 2012). Still today the greatest nuclear and haplotype diversity among modern humans is found in Africa (reviewed by Campbell & Tishkoff 2008), and it is generally accepted that Africa was the source of all current modern human populations. The greatest genetic diversity of North Atlantic bottlenose dolphins is found in the 'pelagic ecotype', and no population structuring has, to date, been found among these dolphins (e.g. Quérouil *et al.* 2007; Mirimin *et al.* 2011, Louis *et al.* 2014a, see Chapter 3). It is therefore likely that the pelagic population is the source population of coastal bottlenose dolphins in the NE Atlantic, as also previously suggested by other authors (Natoli *et al.* 2005; Moura *et al.* 2013; Louis *et al.* 2014b), even though the founding of the coastal NEA habitats may have occurred via the Mediterranean Sea like the evidence in this study indicates. Nevertheless, from the biogeography model, it remains unclear whether the ancestral ranges of present day coastal NE Atlantic lineages tracing back towards deeper nodes in the phylogeny were occupying the pelagic/oceanic habitat or the NW Atlantic

coastal habitats, or both. This was also found by Moura *et al.* (2013), and likely results from restricted amount of samples sequenced from the pelagic population unable to capture all of the lineages. In the future, a greater sampling effort should be concentrated on sequencing a greater proportion of this large, diverse population.

The estimated dates of younger nodes in the phylogeny are concordant with those derived by Moura *et al.* (2013), and are consistent with the geological and climatic context. However, the node date estimates of this study increasingly diverge from the estimates of Moura *et al.* (2013) when going deeper back in time in the phylogeny. In their analysis, Moura *et al.* (2013) used an exponential molecular clock, thus allowing the mutation rate to vary through time, but still struggled to obtain estimates for deeper nodes concordant with fossil calibrations; a fact which the authors fully acknowledged. In this study, both tip and fossil calibrations were applied in combination with using only third codon positions, which are less constrained by purifying selection than the first and second codon positions and expected to evolve in a more clock-like fashion accumulating mutations at a more constant rate, thus minimising the effect of time-dependence. Therefore, the estimate for the splitting of the most divergent clades of bottlenose dolphins in the North Atlantic (800,300 yBP) in this study is nearly twice as old as the estimated split of 486,000 yBP reported by Moura *et al.* (2013). The coalescence time of the Black sea haplotypes BSEA1 and BSEA3 to a Mediterranean haplotype EMED9 also dates back to earlier (ca. 17,400 yBP) than estimated by Moura *et al.* (2013), and the coalescence time of the second Black Sea clade to 16,400 yBP. Our results suggest that the phylogenetic split between the Black Sea and East Mediterranean lineages already occurred before the opening of the Bosphorous Strait and shortly after or during the LGM. There are a number of possible reasons for the difference in the estimates between the two studies. Firstly, biogeographic calibrations are often controversial because of the strong assumption that genetic divergence is tied to geological events. In addition, this process is susceptible to a range of confounding factors, including errors in the estimation of timing of geological events or the degree of association between geological events and genetic divergence (Ho & Duchene 2014). Instead, Ho and Duchene (2014) encourage new and alternative approaches to be applied to geological calibrations, such as tying them to demographic events rather than to genetic divergence events. This reduces the impact of the discrepancy between geographic and genetic divergence, which can be a considerable source of estimation

bias (Edwards & Beerli 2000; Peterson & Masel 2009; Ho *et al.* 2011). Accordingly, the crown age of the BSEA lineages in this study (~6500 yBP) is consistent with a demographic expansion following the opening of the Bosphorous Strait ~10,000 ya.

Other reasons for the differing node age estimates may be attributed to the model selection process. Firstly, calculating Bayes factors with the harmonic mean estimator (HME) is no longer recommended because of its systematic preference for complex models, which can lead to erroneous conclusions (Baele *et al.* 2013). More importantly, Bayes factors were calculated for models with parameters that had not converged (ESS values <200, see Table S6 in Moura *et al.* 2013) and these low ESS values were used instead as a criterion in model selection. In addition, Moura *et al.* (2013) failed to test a strict clock model for the substitution rate variation. In fact, the ucld.stdev parameter is very small (0.013, see Table S6 in Moura *et al.* 2013) in the uncorrelated log-normal relaxed clock model, which indicates a better fit of a strict clock model to the data and in general a clock-like behaviour in the substitution rates between branches (as also found by Duchene *et al.* (2011) in a previous study on delphinids).

Due to its strict maternal inheritance the mitochondrial genome tracks only the matrilineal population history, a very stochastic coalescent process, and has generally less power to detect ancestral demographic changes than estimates based on analyses of multiple nuclear markers (Ho & Lanfear 2008). The fact that the population division of the NE Atlantic samples into 'Coastal North' and 'Pelagic Atlantic' based on nuclear microsatellites (Louis *et al.* 2014a) is reflected in the grouping of the NE Atlantic coastal haplotypes into coastal clades and the pelagic and the Mediterranean/Black Sea samples grouping to their respective clades (Fig. 2.4), suggests that bottlenose dolphin nuclear and mitochondrial phylogenies are at least to some extent concordant, and that the results regarding the phylogenetic history presented in this study are representative of the species tree. However, even though the mtDNA captures most of the samples splitting into pelagic and coastal clades, it fails to capture the finer population structure found among adjacent populations within a coastal area of the NE Atlantic (Louis *et al.* 2014a; Mirimin *et al.* 2011; Chapter 3). This incomplete lineage sorting of the mitochondrial genomes can either reflect a more recent (a few thousand years BP) split between these two coastal populations or a

history of ongoing migration between the demes.

Responses to climate change are likely to vary even among mobile species depending on their tolerance to environmental conditions and the derived habitat preference (Foote *et al.* 2013; Sydeman *et al.* 2015). Data from previous genetic studies on marine mammals have suggested that changes in population structuring and connectivity have followed glacial cycles (Pastene *et al.* 2007; DeBruyn *et al.* 2009; Amaral *et al.* 2012; Foote *et al.* 2013). Dispersal of Southern elephant seals (*Mirounga leonina*), for example, to Victoria Land Coast at Ross Sea, Antarctica, may be linked to increase of available habitat (DeBruyn *et al.* 2009) at the onset of warmer Holocene interglacial (11,700 yBP – present). Similarly, Crandall *et al.* (2012) linked a post-glacial population expansion of marine invertebrates to the timing of sea level rise following the LGM. However, unlike some cold-adapted Arctic terrestrial species that are thought to have gone through range contraction and population bottlenecks whilst living in refugia during interglacials such as the Holocene (Dalén *et al.* 2007; Stewart & Lister 2001), the increase in the effective female population size and genetic diversity of bowhead whales (*Balaena mysticetus*) may have resulted from an expansion of suitable habitat through increased connectivity between the Atlantic and the Pacific, a result of melting of the ice sheets (Foote *et al.* 2013). The bottlenose dolphin, a highly mobile species, is capable of large scale movements but still shows population structure on smaller geographic scales, similar to some other marine mammal species (e.g. Chivers *et al.* 2002; DeBruyn *et al.* 2009; Lowther *et al.* 2012). Overall this study suggests that the present day geographic and genetic structuring of bottlenose dolphins in the North Atlantic were shaped by the climatic cycles of the Late Quaternary, and the most northerly populations represent an expansion by two founding mitochondrial lineages which have rapidly spread throughout, and then retained, the available coastal territory and resources around the British Isles. It also seems likely that the amount of suitable habitat for this species will increase northwards in the next hundred years (Fig. 2.2c), but predicting the consequences of this for populations is difficult. Effective management of biodiversity requires mitigation for the impact of climate change on the distribution of species and populations and an understanding of how they are likely to respond to the inevitable gain or loss of critical habitat in the coming years. Bioclimatic models may provide an

answer to these questions in the face of ongoing directional warming of the Earth's oceans.

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Chapter 2: Appendices

Appendix 2.1 Delphinid mtDNA sequences/mitochondrial haplotypes downloaded from GenBank and used in phylogenetic and coalescent analyses.

Species/ecotype/haplotype	Accession number	Reference
Cephalorhynchus heavisidii	JN632624	Hassanin *et al.* 2012
Orcaella brevirostris	JF289177	Vilstrup *et al.* 2011
Orcaella heinsohni	JF339977	Vilstrup *et al.* 2011
Peponocephala electra	JF289175	Vilstrup *et al.* 2011
Feresa attenuata	JF289171	Vilstrup *et al.* 2011
Globicephala melas	JF339972	Vilstrup *et al.* 2011
Globicephala macrorhynchus	JF339976	Vilstrup *et al.* 2011
Pseudorca crassidens	JF289173	Vilstrup *et al.* 2011
Grampus griseus	EU557095	Xiong *et al.* 2009
Stenella attenuata	EU557096	Xiong *et al.* 2009
Stenella coeruleoalba	EU557097	Xiong *et al.* 2009
Delphinus capensis	EU557094	Xiong *et al.* 2009
Tursiops aduncus	EU557092	Xiong *et al.* 2009
Sousa chinensis	EU557091	Xiong *et al.* 2009
Tursiops australis	KF570363	Moura *et al.* 2013
Lagenorhynchus albirostris	NC005278	Arnason *et al.* 2004
Orcinus orca, resident ecotype	GU187192	Morin *et al.* 2010
Orcinus orca, transient ecotype	GU187173	Morin *et al.* 2010
Steno bredanensis	JF339982	Vilstrup *et al.* 2011
Tursiops truncatus		
EMED3	KF570315	Moura *et al.* 2013
EMED4	KF570316	Moura *et al.* 2013

EMED5	KF570317	Moura *et al*. 2013
EMED1	KF570318	Moura *et al*. 2013
EMED2	KF570319	Moura *et al*. 2013
EMED10	KF570320	Moura *et al*. 2013
EMED6	KF570321	Moura *et al*. 2013
EMED9	KF570322	Moura *et al*. 2013
EMED7	KF570323	Moura *et al*. 2013
EMED8	KF570324	Moura *et al*. 2013
BSEA2	KF570325	Moura *et al*. 2013
BSEA3	KF570326	Moura *et al*. 2013
BSEA1	KF570327	Moura *et al*. 2013
BSEA6	KF570328	Moura *et al*. 2013
BSEA7	KF570329	Moura *et al*. 2013
BSEA5	KF570330	Moura *et al*. 2013
BSEA9	KF570333	Moura *et al*. 2013
BSEA10	KF570334	Moura *et al*. 2013
SCO1	KF570346	Moura *et al*. 2013
SCO6	KF570347	Moura *et al*. 2013
SCO2	KF570348	Moura *et al*. 2013
SCO3	KF570349	Moura *et al*. 2013
SCO4	KF570350	Moura *et al*. 2013
SCO8	KF570351	Moura *et al*. 2013
SCO5	KF570352	Moura *et al*. 2013
WNAC11	KF570370	Moura *et al*. 2013
WNAC13	KF570371	Moura *et al*. 2013
WNAC14	KF570372	Moura *et al*. 2013
WNAC16	KF570373	Moura *et al*. 2013
WNAC19	KF570374	Moura *et al*. 2013
WNAC22	KF570375	Moura *et al*. 2013
WNAC23	KF570376	Moura *et al*. 2013
WNAC25	KF570377	Moura *et al*. 2013
WNAC8	KF570378	Moura *et al*. 2013
WNAP11	KF570379	Moura *et al*. 2013
WNAP12	KF570380	Moura *et al*. 2013
WNAP17	KF570381	Moura *et al*. 2013
WNAP19	KF570382	Moura *et al*. 2013
WNAP21	KF570383	Moura *et al*. 2013

WNAP22	KF570384	Moura *et al.* 2013
WNAP26	KF570385	Moura *et al.* 2013
WNAP7	KF570386	Moura *et al.* 2013
WNAP8	KF570387	Moura *et al.* 2013
WNAP9	KF570388	Moura *et al.* 2013
ENAC1	KT601188	This study
ENAC2	KT601189	This study
ENAC3	KT601190	This study
ENAC4	KT601191	This study*
ENAC5	KT601192	This study
ENAC6	KT601193	This study
ENAC7	KT601194	This study
ENAC8	KT601195	This study
ENAC9	KT601196	This study
ENAP1	KT601197	This study
ENAP2	KT601198	This study
ENAP3	KT601199	This study
ENAP4	KT601200	This study
ENAP5	KT601201	This study
ENAP6	KT601202	This study
ENAP7	KT601203	This study
ENAP8	KT601204	This study
ENAP9	KT601205	This study
ENAP10	KT601206	This study
ENAP11	KT601207	This study

*Haplotype same as SCO4 (and SCO7) in Moura *et al.* (2013), but due to uncertainty associated with haplotype sequences by Moura *et al.* (2013), this haplotype has been sent to GenBank as a new submission.

Appendix 2.2 Best partitioning schemes for substitution models for the construction of the delphinid and North Atlantic *Tursiops truncatus* time-calibrated phylogenies.

Delphinids

Best model	**Subset partitions**
TrNef+I+G	cox1_pos1, cox2_pos1, cox3_pos1, cytb_pos1, nd1_pos1, nd3_pos1, nd4l_pos1, nd6_pos1
HKY+I+G	atp6_pos1, atp8_pos1, atp8_pos2, nd2_pos1, nd4_pos1, nd5_pos1 , atp6_pos2, cox2_pos2, cox3_pos2, cytb_pos2, nd1_pos2, nd2_pos2, nd3_pos2, nd4_pos2, nd4l_pos2, nd5_pos2, nd6_pos2
TrN+I+G	atp6_pos3, cox3_pos3, cytb_pos3, nd1_pos3, nd2_pos3, nd3_pos3, nd4_pos3, nd4l_pos3, nd5_pos3
HKY	cox1_pos2
TrN+G	atp8_pos3, cox1_pos3, cox2_pos3, nd6_pos3

Tursiops truncatus

Best model	**Subset partitions**
TrN+G	cox3_pos3, cytb_pos3, nd1_pos3, nd2_pos3, nd4_pos3, nd4l_pos3, nd5_pos3
HKY	atp6_pos3, atp8_pos3, cox1_pos3, cox2_pos3, nd3_pos3, nd6_pos3

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Chapter 3: Quantifying dispersal between marine protected areas by a highly mobile species, the bottlenose dolphin, *Tursiops truncatus*

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Abstract

The functioning of Marine Protected Areas (MPAs) designated for marine megafauna has been criticized due to the high mobility and dispersal potential of these taxa. However, dispersal within a network of small MPAs can be beneficial as connectivity can result in increased effective population size, maintain genetic diversity and increase robustness to ecological and environmental changes making populations less susceptible to stochastic genetic and demographic effects (i.e. Allee effect). Here, we use both genetic and photo-identification methods to quantify gene flow and demographic dispersal between MPAs of a highly mobile marine mammal, the bottlenose dolphin *Tursiops truncatus*. We identify three populations, two of which have largely non-overlapping core coastal home ranges and are each strongly spatially associated with specific MPAs. We find high site-fidelity of individuals within each of these two coastal populations to their respective MPA. We also find low levels of demographic dispersal between the populations, but it remains unclear whether any new gametes are exchanged between populations through these migrants (genetic dispersal). The population sampled in the Shannon Estuary has a low estimated effective population size and appears to be genetically isolated. The second coastal population, sampled outside of the Shannon may be demographically and genetically connected to other coastal subpopulations around the coastal waters of the UK and the methods applied here should be used on a broader geographically sampled dataset to better assess this connectivity.

3.1 Introduction

The conservation and management of wild animal populations is often implemented through designation of protected areas that are thought to represent important habitats for foraging, breeding and other important activities (Reeves 2000; Palumbi 2001). Demographic connectivity, defined as the linking together of local fragmented populations through the dispersal of individuals as larvae, juveniles or adults (Sale *et al.* 2005), is an important factor to consider when designating marine protected areas (MPAs), since it has implications for the persistence of meta-populations (reviewed in Botsford *et al.* 2009). For example, in many marine fish species, larval dispersal and population connectivity determine whether a MPA (or a network of MPAs) contributes to the overall survival and reproduction of the species, thus maintaining sustainable population sizes (Burgess *et al.* 2014). Dispersal is thus a key variable that conservation biologists need to quantify and consider in order to assess the effectiveness of protected areas (Reeves 2000). This is particularly relevant in highly mobile and wide ranging marine species for which the low cost of movement can facilitate long-range dispersal (reviewed in Forcada 2009), especially considering that the management provision for these kind of species is often restricted to small fixed areas of protection. High levels of mobility can result in substantial gene flow and the homogenization of genetic diversity across a geographic range (e.g. Winkelmann *et al.* 2013). However, whilst in most marine fish meta-populations dispersal during the larval stage facilitates greater connectivity among habitat patches and reduces the risk of local extinctions (Burgess *et al.* 2014), marine mammals typically have much lower reproductive rates and their offspring can exhibit a high degree of natal philopatry (Baird 2000; Sellas *et al* 2005; Amos *et al.* 1993). This can lead to small isolated populations and a system that is sensitive to changes in environmental conditions, ecological factors or anthropogenic disturbance.

Lowe and Allendorf (2010) distinguished demographic connectivity from genetic connectivity by defining the former as the relative contribution of net immigration and local recruitment to the population growth rate, and the latter as the degree to which evolutionary processes within (sub)populations are affected by gene flow. Population genetic approaches may provide a tool to measure and quantify the rate and scale of dispersal (i.e. migration) when it is not feasible to assess the movement of individuals

by non-genetic capture-recapture methods (Gagnaire *et al.* 2015), but when combined together, genetic and non-genetic methods are highly complementary and can provide invaluable information for management of populations. Photo-identification is a cost effective technique commonly used by marine mammal researchers to identify individuals of many species using the unique natural markings on their body and thus enabling, for example, the estimation of their distribution, association patterns or abundance via capture-recapture methods (see review by Würsig & Jefferson 1990). If natural markings cannot be used because of insufficient individual variation, molecular genotyping may provide a usable (albeit more costly) alternative to photo-identification methods in estimating animal movements (see Palsbøll *et al.* 1997). Here, both these approaches were applied to quantify the demographic and genetic connectivity between marine protected areas designated for bottlenose dolphins in an area in the north-east Atlantic.

Bottlenose dolphin species are widely distributed, being found in the Atlantic, Indian and Pacific oceans (Leatherwood & Reeves 1990). Throughout much of its range, the common bottlenose dolphin (*Tursiops truncatus*) exhibits hierarchical population structure, with the greatest divergence found between pelagic and coastal populations (Curry & Smith 1998; Hoelzel *et al.* 1998; Louis *et al.* 2014a,b; Lowther-Thieleking *et al.* 2015). Genetic differentiation is often correlated with ecological and/or morphological differences (Hoelzel *et al.* 1998; Louis *et al.* 2014a; Natoli *et al.* 2004; Hersh & Duffield 1990). Further fine-scale structuring has then been found among coastal populations in several locations (Natoli *et al.* 2005; Parsons *et al.* 2002, 2006; Baird *et al.* 2009; Rosel *et al.* 2009; Fernández *et al.* 2011; Martien *et al.* 2011; Mirimin *et al.* 2011; Caballero *et al.* 2012; Gaspari *et al.* 2013, 2015; Louis *et al.* 2014a,b; Martinho *et al.* 2014). The driving forces behind fine-scale population structuring among coastal populations of bottlenose dolphins are not fully resolved, but have been suggested to include isolation following a historical founding event (see Chapter 2), habitat preference, differences in social structure and site fidelity, learned foraging specializations, natal philopatry, limited dispersal of both sexes, and habitat discontinuity linked to prey availability (Natoli *et al.* 2005; Krützen *et al.* 2004b; Rosel *et al.* 2009; Gaspari *et al.* 2015; Louis 2014b Krützen *et al.* 2004a; Parsons *et al.* 2006; Louis 2014a,b; Martien *et al.* 2012).

Common bottlenose dolphins are listed in Annex II of the European Union's Habitats Directive requiring the member states to designate Special Areas of Conservation (SACs) as part of an overall European strategy (Natura 2000) to maintain or restore the species at "favourable conservation status". Consequently, SACs (or Natura 2000 sites) have been designated in the coastal waters of several areas in EU Member States. In the NE Atlantic such SACs are located in Moray Firth in Scotland, Cardigan Bay in Wales, Sado Estuary in Portugal, Iroise Sea in France (two protected areas), and in two areas on the west coast of Ireland; the Shannon Estuary and in western parts of Counties Galway and Mayo (West Connacht Coast) (see Fig. 3.1). However, what these SACs represent in relation to bottlenose dolphin habitat use, population connectivity and social structure is unclear.

Bottlenose dolphins using the Shannon Estuary SAC have been found to be genetically differentiated from another population inhabiting the coastal waters off counties Galway and Mayo (Mirimin *et al.* 2011). However, these findings were based on a limited number of samples collected in a relatively small area (ranging about 70km along the Galway/Mayo coastline) and it is not known whether additional structuring exists. Photo-identification studies of dolphins using the Shannon Estuary SAC suggest that these individuals have a high degree of site fidelity (e.g. Ingram & Rogan 2003; Englund *et al.* 2008), however, the extent of the range of dolphins using Ireland's coastal waters is not yet fully understood. Previous research has shown that at least some of these coastal animals move over great distances (Ingram *et al.* 2001, 2003; O'Brien *et al.* 2009; Oudejans *et al.* 2010; Robinson *et al.* 2012; Cheney *et al.* 2013), which could indicate some potential for genetic connectivity between adjacent sub-populations using neighbouring coastal SACs, but this has not previously been demonstrated or quantified.

Genetic clustering and kinship-based methods are used here to re-examine the population structure in Irish waters using a larger dataset supplemented with samples collected from a wider coastal area. The contribution of demographic and genetic dispersal to the connectivity between neighbouring SACs within Irish waters are quantified using a combination of photo-identification and genetic techniques. In addition, the role of possible drivers for population structuring, including social structure, relatedness, site-fidelity and sex-biased dispersal are examined. The findings are discussed in the context of conservation and management and, more specifically,

the effectiveness of networks of SACs for sustaining the viability of local demes and the meta-population as a whole.

3.2 Materials and Methods

Photo-identification surveys and photograph selection

Boat-based photo-identification surveys were conducted within the Lower River Shannon SAC, Ireland, every year between 1996 to 2008 with the exception of 2004, and in other coastal areas of Ireland (including the West Connacht Coast SAC), in 2001-2005, 2007-2010 and 2013-2014 (Figs. 3.1 and 3.2). A bottlenose dolphin 'group' was defined as all dolphins within a 100m radius of each other as per Irvine *et al.* (1981) and hereafter 'encounters' refer to periods of data collection whilst with dolphin groups. Best effort was made to attempt to photograph every individual in the group, and identification photographs of bottlenose dolphins' dorsal fins were processed following methods described by Englund *et al.* (2007). For each encounter, the best quality photograph was chosen of each identifiable dolphin and the quality of the photograph was graded from 1 to 4 (1 being the highest quality, 4 being the lowest) with no consideration concerning the degree of marking of the individual. Each photographed individual was then assigned one of three grades of mark-severity (Fig. 3.3), and visually matched against the full catalogue of dolphins photographed during previous encounters.

Skin tissue sample collection and analysis

The dataset comprising of altogether 97 unique samples included in total of 85 samples already genotyped by Mirimin *et al.* (2012). This set of 85 genotypes included 45 skin tissue samples collected from animals in the Shannon Estuary SAC in 2005 and 2007, four samples from animals encountered in Cork Harbour in 2008 and 12 samples collected from animals ranging in coastal waters of Galway and Mayo (part of West Connacht Coast SAC) during 2009 (Fig. 3.1). The previously genotyped dataset also

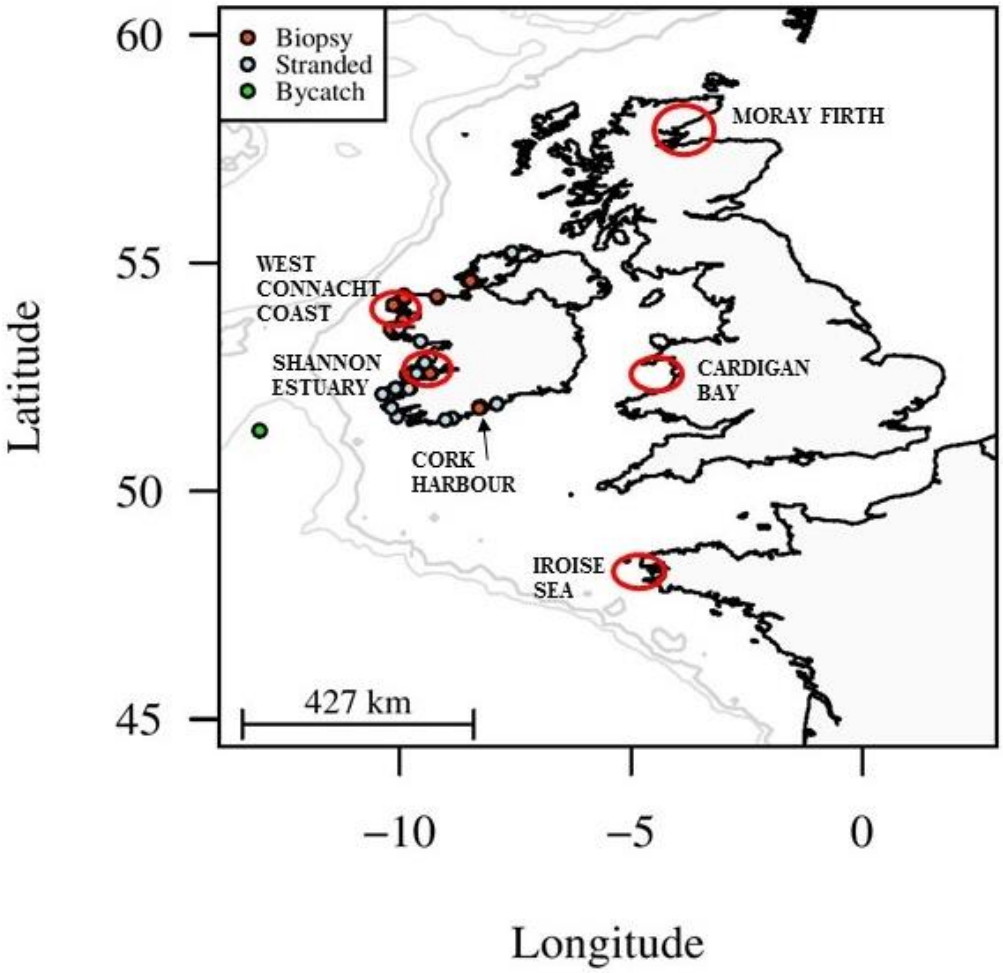

Figure 3.1 GPS-locations of bottlenose dolphin samples collected and used throughout this study and approximate locations of Special Areas of Conservation (SACs) in NE Atlantic (areas circled). Samples include coastal biopsies of free-living dolphins (N = 71), samples collected from dead stranded animals (N = 25) and one sample from a by-caught animal. Note that some sampling locations indicated by the circles overlap due to the scale of the map.

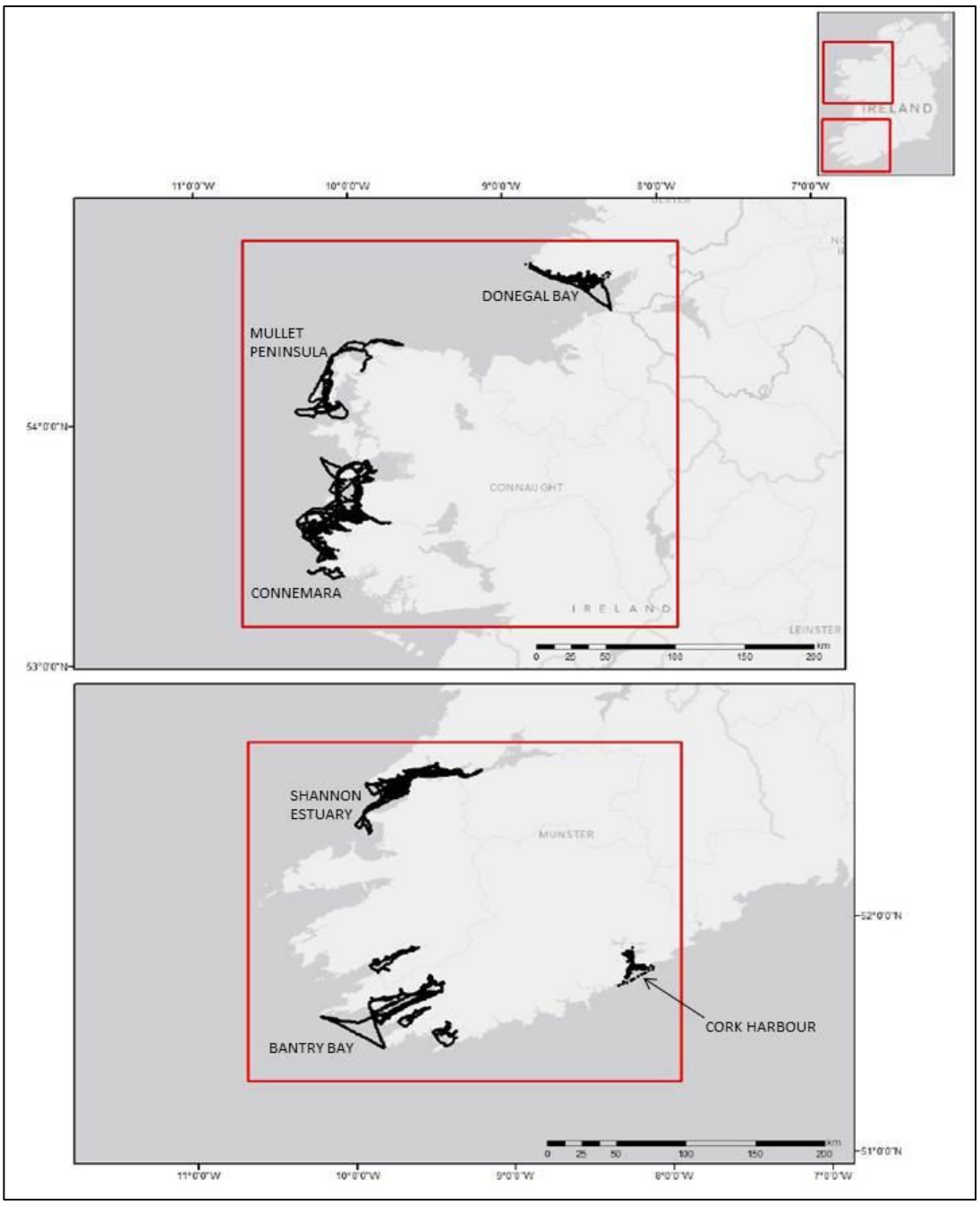

Fig 3.2 GPS tracks recorded during boat surveys for bottlenose dolphins on the West coast of Ireland.

Figure 3.3 Examples of bottlenose dolphin fins showing the three grades of mark severity used in photograph analysis. Each dolphin was graded from one to three as follows: (A) grade M1 marks, consisting of significant fin damage or deep scarring that were considered permanent; (B) grade M2 marking that consist of deep tooth rakes and lesions, with only minor cuts present; (C) fin with grade M3 marks, having only superficial rakes and lesions. Grade M1 and M2 are considered to last many years, enabling long-term identification of these dolphins. In contrast, 'superficial' markings (grade M3), such as tooth rakes may fade and heal within a relatively short period of time and inter-annual re-sighting probabilities of these animals are likely to be reduced.

included samples collected from 23 individuals stranded along the west coast of Ireland, including two dolphins found dead within the Shannon Estuary, between 1993 and 2009. This dataset was supplemented by ten skin biopsies collected from free-ranging animals in coastal waters of Co. Mayo and Co. Donegal during 2013-2014, a sample from a dolphin that stranded in Co. Cork in 2014, and a sample collected from an animal that was by-caught by a fishing vessel on the continental shelf off south-west of Ireland in 1996. All of the skin biopsy samples in this study were taken using a modified rifle (see Krützen *et al.* 2002) and sampling was carried out during the summer months. The gender of stranded individuals was recorded by inspection of the genital area and reproductive organs, while sex of free-ranging biopsied individuals was determined by multiplex amplification of sex chromosome-specific DNA fragments, following the method described in Rosel (2003).

DNA extraction, PCR amplification and genotyping

DNA was extracted from skin samples using the DNeasy Blood and Tissue kit from Qiagen. A total of 15 nuclear microsatellite loci (see Appendix 3.1) were amplified following polymerase chain reaction (PCR) conditions described in Mirimin *et al.* (2012). The amplified products were separated on 6% polyacrylamide gels on a LICOR 4300 DNA analyser (Li-Cor Inc, Lincoln, NE, USA) and allele sizes determined by eye in comparison to a 50–530 size standard (LI-COR) and allele cocktails from reference samples. To allow comparison with previously published

data, new samples collected for this study (N = 12) were genotyped following the protocol described in Mirimin *et al.* (2012). Due to the fact that it is not uncommon to biopsy sample the same individual dolphin more than once, the uniqueness of the new genotypes was confirmed by calculating the percentage of similarity between the samples in program GIMLET 1.3.3 (Valière 2002). The same program was also used to calculate the probability of identity (PI), which estimates the power of the set of microsatellite markers to resolve between two distinct individual samples (Waits *et al.* 2001). The error rate involved in genotyping had already been estimated as negligible (< 0.01%) by Mirimin *et al.* (2012), therefore, re-estimation of the error was not performed for the new samples because of their low number (N = 12).

The 15 microsatellite loci were checked for null alleles, allelic dropout and stuttering, using MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004) and selecting the Bonferroni adjusted 95% confidence interval option with 1,000 simulations. Additionally, MICRODROP 1.01 (Wang *et al.* 2012) was used to further check for allelic dropout due to low DNA concentration or poor sample quality. The microsatellite loci were inspected for significant deviations from Hardy-Weinberg equilibrium (HWE) using GENEPOP (Raymond & Rousset 1995; Rousset 2008) and linkage equilibrium using ARLEQUIN (Excoffier & Lischer 2010) with 10,000 iterations and applying sequential Bonferroni corrections. The above analyses were performed considering the whole dataset as a single unit and also separately at population level (identified with Bayesian clustering methods, see below).

Individual assignment tests

All samples were included in a cluster analysis using STRUCTURE (Pritchard *et al.* 2000). The admixture model was run with correlated allele frequencies without including any prior information on the sampling location. Ten independent runs were carried out for each value of K (the number of theoretical populations), with K set to vary from 1 to 6, using 1,000,000 Markov Chain Monte Carlo (MCMC) iterations preceded by 1,000,000 burn-in steps. Convergence of chains (traces of alpha and F_{ST} values) and the consistency of runs were checked by confirming that the variance in estimated ln Pr(X|K) was smaller within each K compared to the variance between the different Ks, and calculating the average posterior probability for each K. ΔK, which has been argued to be a better predictor of the number of populations, was also

calculated following Evanno *et al.* (2005) in STRUCTURE HARVESTER web-version 0.6.94 (Earl & vonHoldt 2012). Once *K* was determined, each individual was assigned to a cluster based on its maximum membership proportion.

Since relatedness between individuals can affect population assignment (i.e. including samples of closely related individuals can lead to artificial structuring of populations (Guinand *et al.* 2006; Anderson & Dunham 2008), the relatedness coefficient, *r*, (Queller & Goodnight 1989) was calculated between all possible dyads within the putative populations identified by the clustering methods using KINGROUP (Konovalov *et al.* 2004). Subsequently, one member of each dyad with a relatedness coefficient of 0.45 or greater was removed (according to Rosel *et al.* 2009) and STRUCTURE re-run with this reduced dataset.

In addition, population structuring was inferred using a discriminant analysis of principal components (DAPC) that clusters individuals together based on genetic similarity to find the most likely number of populations. DAPC does not rely on any population genetic model (i.e. does not assume HWE) and is efficient at detecting hierarchical structure (Jombart *et al.* 2010). DAPC using the package adegenet (Jombart 2008) in R (R Core Team 2016) was run, and cluster membership probabilities was calculated for each individual.

A third clustering method was implemented in program TESS (Durand *et al.* 2009a,b) which uses GPS-coordinates along with genetic markers in order to infer population structure; therefore only biopsy samples were used in this analysis since stranded and by-caught individuals had unknown geographic origins. The conditional autoregressive (CAR) model was used with admixture using 20,000 burn-in followed by 120,000 MCMC steps with the number of clusters, *K*, varying 2–10, with 10 replicates per run. The most probable number of clusters was selected by plotting Deviance Information Criterion (DIC) values against different values of *K* and by examining individual assignment probability plots. Consistency of the runs was checked by examining the convergence of MCMC chains in TRACER 1.6. (Rambaut *et al.* 2014). TESS cannot directly test for $K = 1$ but we checked this by examining individual assignment probabilities. When the most likely *K* was determined, the run with the lowest DIC was used and individuals were assigned to clusters based on maximum assignment probabilities.

The results from all clustering methods (see below) were consistent in their inference of the most likely number of clusters and mostly consistent in the individual assignment probabilities so the data set was divided into three putative populations, *Coastal Shannon*, *Coastal mobile* and *Pelagic*, for the remaining genetic analyses. There is uncertainty associated with the geographic range of the *Pelagic* population since the samples consist mostly of stranded animals, but based on the fact that these animals have not been photographed in coastal waters coupled with their genetic divergence, and for consistency with previous publications e.g. Louis *et al.* (2014a), this population is referred to as the *Pelagic* population.

Population differentiation was estimated by calculating pairwise F_{ST} (Weir & Cockerham 1984) and Jost's *D* (Jost 2008) values using the R package diveRsity (Keenan *et al.* 2013) between populations identified by STRUCTURE, with the whole and the reduced dataset after the removal of close relatives, and the 95% confidence intervals were obtained using 10,000 bootstrap replicates. Population specific F_{IS}-values, expected and observed heterozygosity, number of alleles (uncorrected) and allele richness (corrected for sample size) were also calculated using package diveRsity in order to examine the level of inbreeding. Heterozygote deficiency and excess in each population was tested using Fisher's method implemented in GENEPOP (Raymond & Rousset 1995; Rousset 2008) with 10,000 iterations. As a further check that differentiation was not solely driven by sampling of related individuals or uneven sampling of populations (see Puechmaille 2016), 10 individuals were randomly selected from each of the two putative coastal populations and the pairwise F_{ST}-values (with 95% CI) estimated using the R package diveRsity and repeated 10 times. These pairwise values were compared to F_{ST}-values calculated for two sets of ten individuals randomly drawn from within a single coastal population, *Coastal Shannon* or *Coastal mobile*. To supplement this analysis, the power to detect a significant moderate population differentiation based on F_{ST} value of ≥ 0.1 in a sample consisting of the allele frequencies from both coastal populations and using a sample size of ten individuals per 'subpopulation' (i.e. *Coastal Shannon* and *Coastal mobile*) was calculated by running 1,000 simulations in POWSIM 4.1 (Ryman & Palm 2006, see also Ryman *et al.* 2006; Morin *et al.* 2009).

Sex-biased dispersal between the three populations identified by clustering methods was tested by comparing the mean and variance of log-transformed corrected assignment indices (mAIc and vAIc) and F_{ST} and F_{IS} statistics separately for males and females between the populations, using 1,000 permutations with R-package 'hierfstat' (Goudet 2005). Following Goudet *et al.* (2002), it was assumed that sex-biased dispersal from the sampled populations could be detected from gender differences in genetic structuring with the more philopatric sex showing more structure and a significantly negative mAIc.

Migration rates

Recent migration rates (proportion of migrants per population) within the last two generations were estimated using BAYESASS (Wilson & Rannala 2003). The migration rates were calculated between the populations identified by STRUCTURE and DAPC, and then re-estimated with the individual biopsied in the Shannon Estuary but genetically assigned to *Coastal mobile* population grouped together with the Shannon dolphins. The MCMC mixing parameters of migration rates, allele frequencies and inbreeding coefficients, were adjusted as recommended by Rannala (2007), during preliminary runs in order to obtain proposal acceptance rates of around 30%. Ten runs with a burn-in of 1,000,000 iterations followed by 10,000,000 MCMC iterations sampling every 1,000 iterations were performed. Convergence and mixing of chains were checked by plotting trace files using TRACER (Rambaut *et al.* 2014) and for consistency of runs.

Effective population size

An estimate of contemporary effective population size (N_e) for the *Coastal Shannon* population was derived using LDNe, a method that uses linkage disequilibrium (Waples & Do 2008). This method has performed best in situations with little to no migration (<1%) (Gilbert & Whitlock 2015) and adequately with migration rates of up to ~5–10% (Waples and England 2011). Allele frequencies of <0.02 were excluded from the analyses to avoid bias caused by rare alleles (Waples & Do 2010; Louis *et al.* 2014a), and since the samples were collected over a 15-year time period (in the Shannon estuary) and the data are thus likely to be biased downwards due to overlapping generations (Waples 2010), the estimate of N_e was inflated by 15% as in Louis *et al.*

(2014a). N_e could not be calculated for the *Coastal mobile* and *Pelagic* populations, due to small sample size (Tallmon *et al.* 2010).

Analyses of social structure and site fidelity

To test possible drivers of population structure and connectivity, social structure, site fidelity and kinship was examined among the coastal bottlenose dolphins (*Shannon* and *Mobile*); long-term photo-identification data are not available for the 'pelagic' dolphins in this area. Social structure analyses were performed in SOCPROG 2.4 compiled version (Whitehead 2009). The dataset was limited to individuals with permanent and obvious markings (mark severity grade M1, Fig. 3.3) in order to identify individuals between several years, and only dolphins photographed in at least five separate encounters were included to reduce bias caused by rarely seen individuals (Whitehead 2008). Individuals photographed together during an encounter were considered associated with each other, so an encounter was chosen as the grouping variable in SOCPROG. "Day" was chosen as the sampling period.

The strength of association between pairs of individuals (i.e. dyads) was measured using two indices of the frequency of co-occurrence: the half-weight association index (HWI) and the simple ratio (Cairns & Schwager 1987; Ginsberg & Young 1992). The simple ratio index is suitable when association is defined by presence in the same group during a sampling period (Ginsberg and Young 1992), however, the half-weight index (HWI) can be more appropriate when not all individuals within a group have been identified, as is often the case with dolphin photo-identification studies due to individuals reacting differently to the presence of the research vessel. Since both indices gave almost identical results and were considered good representations of social structure by the high cophenetic correlation coefficient (ccc) values (ccc HWI: 0.874, ccc simple ratio: 0.887), only the results derived using the HWI are presented. NETDRAW (Borgatti 2002) was used to visualize a social network diagram using the network statistics calculated in SOCPROG. Permutation tests (Bejder *et al.* 1998; Whitehead 1999) with 20,000 steps were used to test whether the observed association patterns were different than expected from random associations, and to identify dyads with significantly larger or smaller association indices than expected from randomly associated individuals.

The standardized lagged association rate (SLAR) was used to test if temporary or long-lasting social bonds existed between individuals, and compared to the null association rate (expected if all individuals are associating at random). The SLAR was fitted separately to the individuals encountered within and outside of the Shannon Estuary since the data showed that these groups did not associate with each other. Mathematical models representing simulated social structures (Whitehead 1995) were fitted to the SLARs. The best-fitting models were chosen based on the lowest quasi Akaike's Information Criterion (QAIC) value (see Whitehead 2007). To investigate movements of dolphins between different coastal areas and to estimate the amount of time identified individuals resided within each area, Lagged Identification Rates (LIRs) within and between all study areas were calculated in SOCPROG 2.4 (Whitehead 2009). Markov movement models (expected LIRs) of emigration/mortality and emigration + re-immigration (Whitehead 2001) were fitted to estimate the probabilities of individuals moving from one area to another, and QAIC-values were used to identify the best fitting model. 100 bootstrap replicates were used to estimate the standard error for the LIRs.

Relatedness, associations and spatial overlap

A Mantel-test in R-package ade4 (Dray and Dufour 2007) was used to investigate whether associations reflected kinship bonds, and whether a correlation existed between the strength of pairwise association (HWI) and relatedness between all biopsied dyads that had been encountered at least three times. To examine whether there was a correlation between spatial overlap and relatedness kernel utilization distribution (KUD) was calculated for individually identified dolphins that were encountered at least five times using R-package adehabitatHR (Calenge 2006), and the overlap in the areas used by two dolphins was then estimated by calculating the volume of intersection (VI) index (Podgórski *et al.* 2014; Fieberg & O'Kochanny 2005) of KUD. This index takes values between 0 and 1, and it quantifies the similarity between two KUDs thus comparing the area shared and the intensity of use by two individuals. These correlation tests were performed for the combined dataset and also separately for each of the two coastal populations, and significance tested in the correlations by performing randomization tests with 10,000 MCMC permutations.

3.3 Results

Twelve new individuals were genotyped for this study and analysed together with 85 previously genotyped unique individuals from Mirimin *et al.* (2011). Including the whole dataset of 97 genotypes, the number of missing alleles was 54 thus representing <2% of the total of 2910 amplified alleles. The probability (PI) of two of the 97 individuals sharing the same genotype over the 15 microsatellite loci was 4.5×10^{-14} for any two random unrelated individuals and 5.9×10^{-6} for siblings. This indicates that the set of markers used in this study has high power to discriminate between identical genotypes (i.e. if the same animal was sampled twice, or identical genotypes arising from of chance alone). No identical genotypes were found among the new samples genotyped in this study. When all the populations identified by clustering methods were tested for deviations from HWE across all microsatellite loci, eleven out of the fifteen loci were found to be out of HWE. Further tests using MICRODROP indicated no correlation between the amount of homozygotes and the amount of missing data across individuals (Pearson r = -0.091, P = 0.85) or across loci (Pearson r = 0.178, P = 0.26), suggesting that homozygosity was not due to allelic dropout. Therefore, the observed deviations from HWE across all populations and loci are most likely attributed to the structuring of the populations, i.e. Wahlund effect (Wahlund 1928). When deviations from HWE were inspected for each population separately, only two loci (*Dde*66 and *Dde*72) within the *Coastal mobile* population and one locus (*Dde*61) within the *Pelagic* population were out of HWE (Appendix 3.1). STRUCTURE was therefore run with and without these three loci.

Individual assignment tests

The most likely number of clusters (i.e. populations), K, identified by STRUCTURE based on the highest Pr($X|K$) and using the *ad-hoc* method by Evanno *et al.* (2005) was three when all the coastal biopsies and stranded samples were included in the analysis (Fig. 3.4a). The majority of the individuals (92 out of 97) were strongly assigned (with probability >90%) to one of these three clusters (Fig. 3.5a). Removing the three loci that were out of HWE did not have an effect on the most likely number of clusters or the assignment of individuals into the three clusters. However, when considering assignments at K = 2, the *Coastal mobile* dolphins clustered together with the *Pelagic* dolphins with high (>80-90%) assignment probabilities instead of clustering together

with the *Coastal Shannon* as was the case when all loci were included (latter presented in Fig. 3.7a). This may have resulted from the large number of unique alleles only found in the pelagic samples (altogether 13 unique alleles) being left out from the analysis.

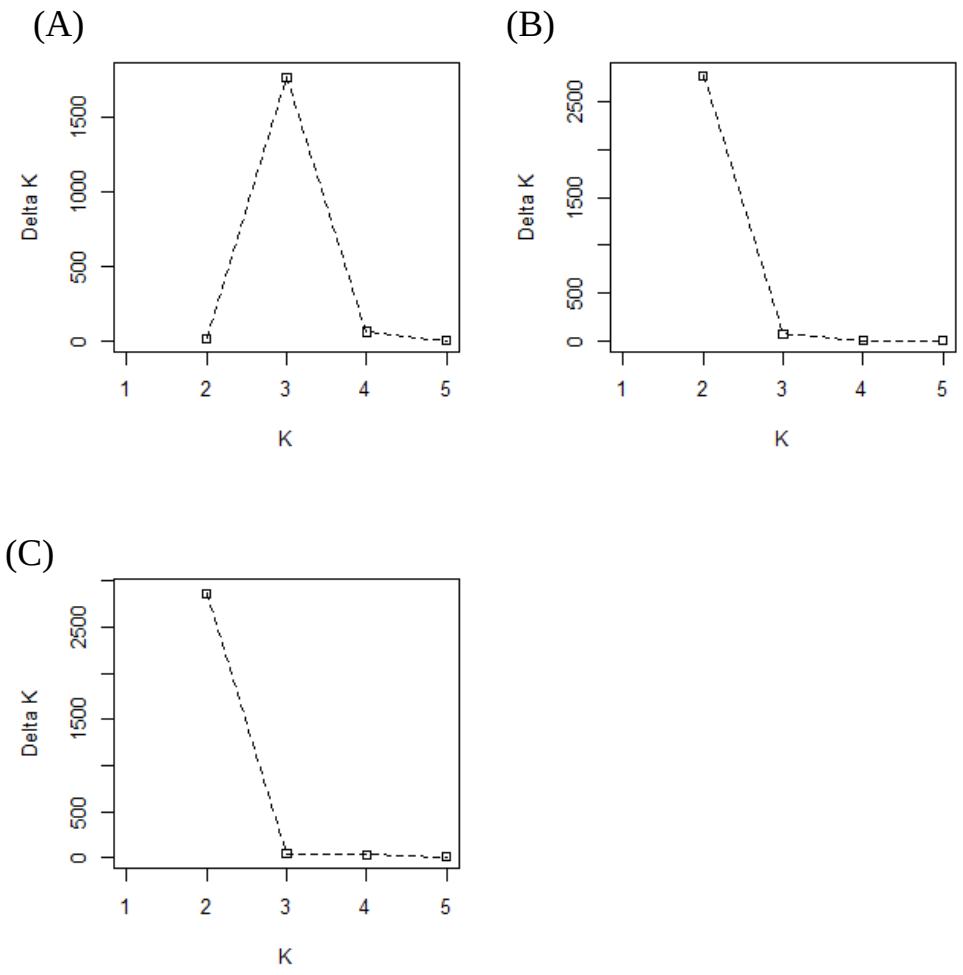

Figure 3.4 Second order rate of change for mean log-likelihood, ΔK (i.e. Evanno-method), for different number of populations, *K*, (A) including all samples, (B) only coastal samples (*Shannon* and *mobile*) included, and (C) only coastal samples included and close relatives ($r \geq 0.45$) removed from the data set.

One individual (DNA sample code 'tt-05-03' and photo-id number 18, see Fig. 3.6) biopsy sampled inside the Shannon Estuary was assigned to the *Coastal mobile* cluster with 79% probability by STRUCTURE (individual indicated in Fig. 3.5a; and in Fig. 3.6, as a possible migrant; this was also found by Mirimin *et al.* (2011). Four dolphins sampled in Cork harbour were strongly assigned (>80% probability) to the same cluster with the *Coastal Shannon* dolphins (Fig. 3.5a and Fig. 3.6), consistent with Mirimin *et al.* (2011). Two individuals found dead-stranded outside of the Shannon estuary (~30km and ~50km north of the mouth of the estuary) were assigned to the *Coastal Shannon* population (Fig. 3.5a); this may be a result of carcass drifting or an indication that at the least some of the *Coastal Shannon* population are using areas beyond the estuary.

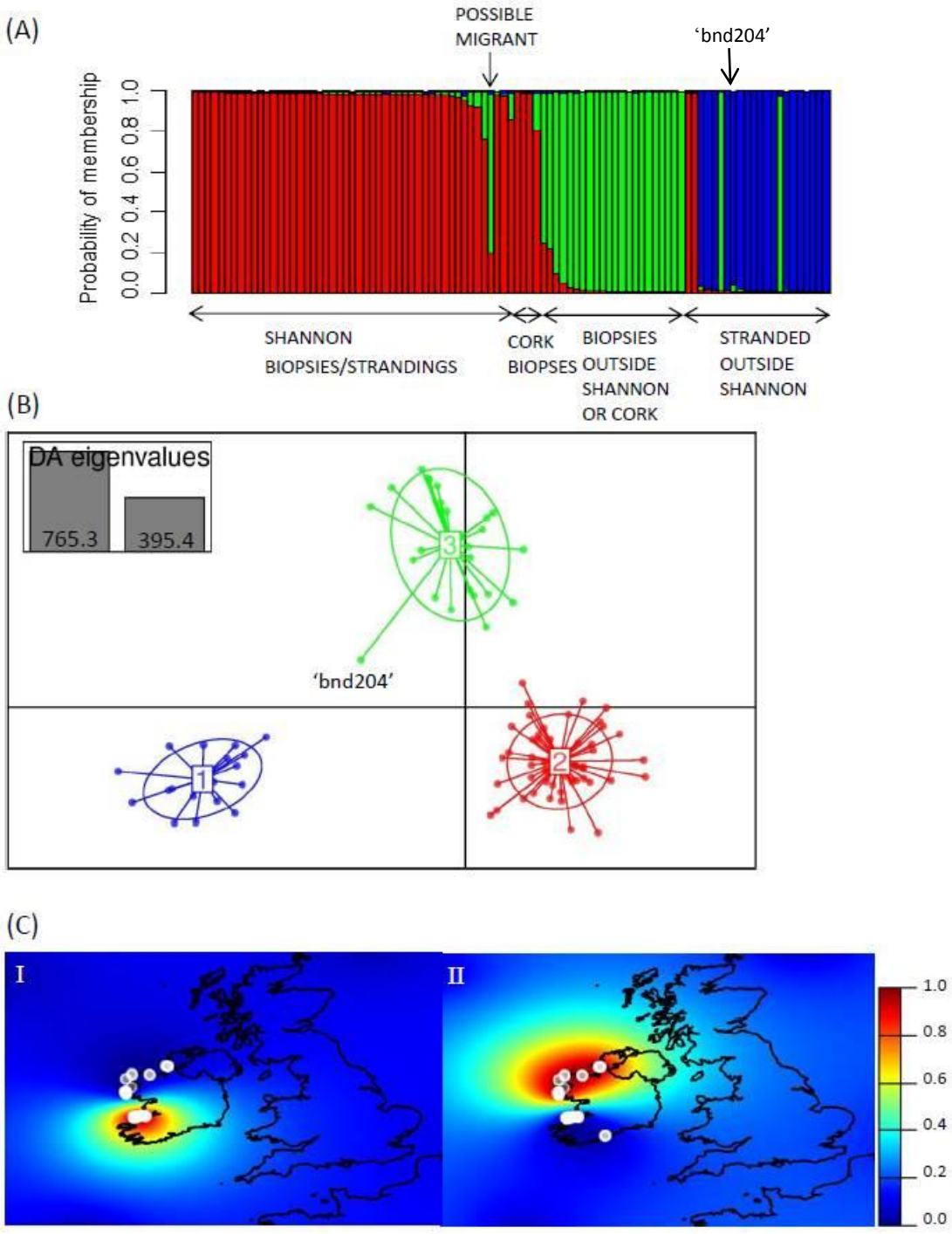

Figure 3.5 (A) Genetic assignment probabilities from STRUCTURE (*N* = 97) with each vertical column corresponding to an individual dolphin and the colours indicating the membership proportions to each of the three clusters. (B) DAPC scatterplot clustering the samples (*N* = 97) according to their first two principal components. The outlier 'bnd204' was the only sample assigned differently by DAPC and STRUCTURE. Red, green and blue colours represent *Coastal Shannon*, *Coastal Mobile* and *Pelagic* dolphins, respectively. (C) Map of individual assignment probabilities per population (I) *Coastal Shannon* (II) *Coastal mobile* identified by TESS including only coastal biopsies (*N* = 71) symbolized with white dots. The colour scale bar indicates the assignment probabilities. The results are based on analyses run with the complete set of 15 microsatellite loci.

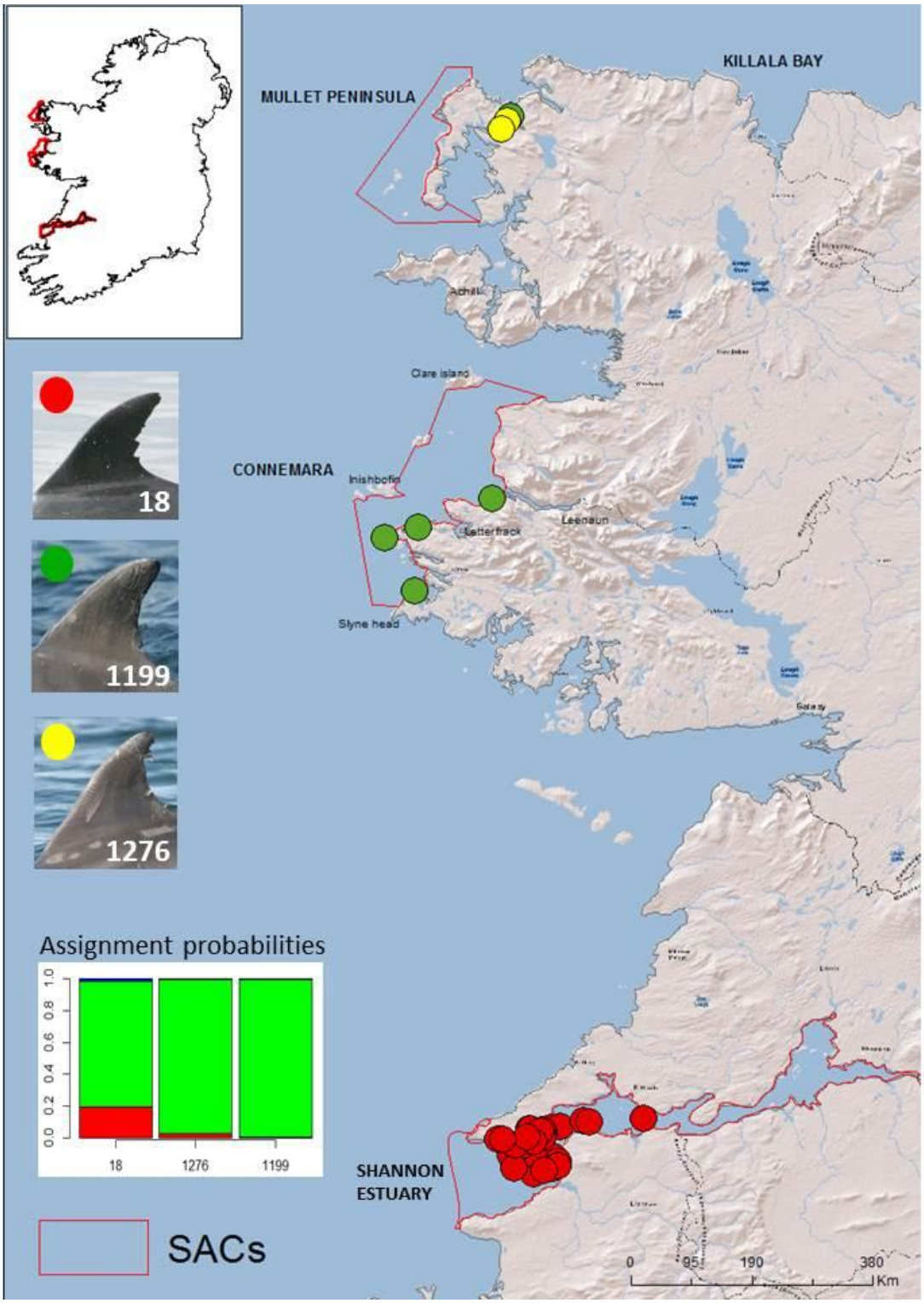

Figure 3.6 Possible migrant dolphin (a male given photo-ID number 18) has been encountered only within Shannon estuary SAC over 9 years (encounter locations indicated with red dots) but is genetically assigned to coastal mobile population with 79% certainty (green colour in assignment probability plot from STRUCTURE). Dolphin 1276 (encounter locations indicated with green dots) is a male potentially closely related to18 ($r \geq 0.45$), and he in turn is closely related to 1199 (encounter locations indicated with yellow dots), also a male. Both 1276 and 1199 are strongly assigned to the coastal mobile population.

DAPC, which does not assume HWE, also identified three clusters when all the samples were included (Appendix 3.3) with a mild hierarchical structure among them; the distance between the clusters of *Coastal Shannon* and *Coastal mobile* samples is shorter than the distance between either of the coastal clusters and the *Pelagic* cluster (Fig. 3.5b). Individual assignments were high (>99%) and highly consistent compared to STRUCTURE with 99% of the individuals assigned to the same cluster across the methods. In fact, only one stranded individual (sample code 'bnd204', an outlier in Fig. 3.5b) was assigned to the *Coastal mobile* cluster by DAPC whereas it was clustered together with stranded pelagic samples by STRUCTURE when all the samples were included (Fig. 3.5a). Similarly, in STRUCTURE, when close relatives were removed from the dataset, the sample 'bnd204' clustered together with *Pelagic* samples with $K = 2$, and with *Coastal mobile* samples when K was set to 3 (Appendix 3.2).

These results were consistent with clustering probabilities calculated in TESS when only the biopsy samples of coastal dolphins ($N = 71$) were considered; the most likely number of coastal populations identified was two (Fig. 3.5c, see also Appendix 3.5) as indicated by the DIC-values reaching a plateau (Appendix 3.4). The individual assignment probabilities were also 100% consistent with STRUCTURE and DAPC with all the same individuals assigned with >90% probability to either the *Coastal Shannon* or the *Coastal mobile* cluster (excluding the individual sampled in the Shannon Estuary that assigned to the *Coastal mobile* cluster with 59% certainty).

The samples assigned to the *Coastal Shannon* population had the largest percentage (2.4%) of dyads that were close relatives, with the Queller and Goodnight (1989) relatedness coefficient $r \geq 0.45$ indicating possible parent-offspring or full sibling relationships among these individuals. Relatedness was also found in the *Coastal mobile* cluster, with 2.0% of all possible dyads assigned as being close relatives; no close relatives were found among the *pelagic* samples. The mean relatedness coefficient varied from -0.02 (SD = 0.23) among individuals assigned to the *Coastal Shannon* population, -0.04 (SD = 0.25) among the *Coastal mobile*, to -0.06 (SD = 0.13) among the *Pelagic* dolphins. The mean relatedness values within the *Coastal Shannon* (1431 possible dyads) and the *Coastal mobile* (300 dyads) were also significantly

higher compared to the relatedness of dyads when individuals were selected one from each of the two coastal populations (1350 dyads, Kruskal-Wallis $P < 0.0001$).

Removing one individual from a dyad with relatedness coefficient $r \geq 0.45$ led to the removal of 22 individuals from the *Coastal Shannon* and six individuals from the *Coastal mobile* dataset. When considering only these 'coastal' samples, the most likely number of clusters identified by STRUCTURE was still two (Figs. 3.4b and 3.4c) and the majority of individuals (46 out of 48) were assigned to either of the two coastal populations with >80% certainty (Fig. 3.7).

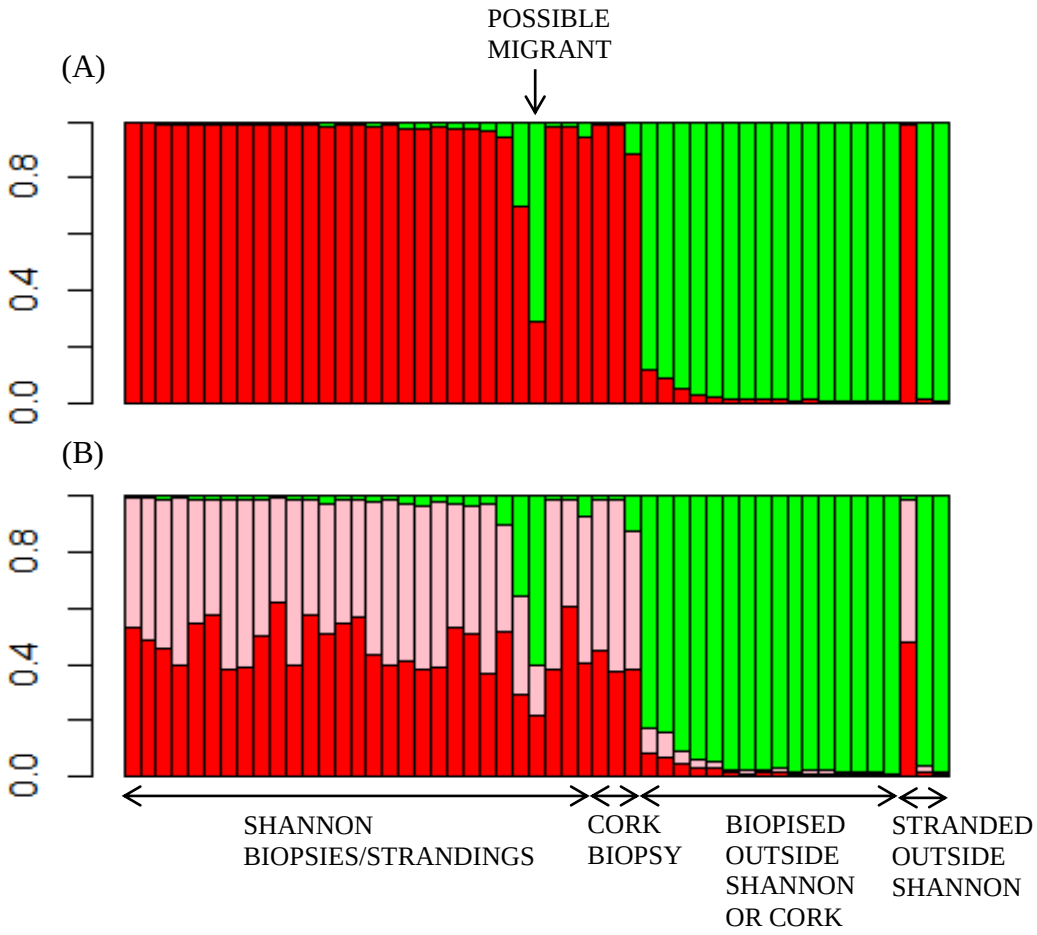

Figure 3.7 Population assignment probabilities (STRUCTURE) of coastal Irish bottlenose dolphins (*Shannon* and *mobile*) after removal of close relatives ($r \geq 0.45$) from the dataset, with (A) $K = 2$, and (B) $K = 3$, where K is the candidate number of populations. Each vertical column corresponds to an individual dolphin.

Population differentiation and effective population size

No evidence of significant heterozygote deficiency was found across all loci in any of the populations (*Coastal Shannon P* = 0.998, *Pelagic P* = 0.469, *Coastal mobile P* = 0.061). Allele richness (AR) and observed heterozygosity (H_O) were lower in the two *coastal* populations compared to the *pelagic* population (Appendix 3.1). Inbreeding coefficients were low in all populations. The mean estimate for effective population size in the *Coastal Shannon* population was 32 (with 95% CI of 22 – 43).

There was significant differentiation in allele frequencies (based on both F_{ST} and Jost's *D*) in all comparisons between the *pelagic* and the two *coastal* populations, and this difference persisted after removing close relatives from the dataset (Table 3.1). The Jost's *D* values revealed a hierarchical population structure, with largest differences observed between the *pelagic* and the two *coastal* populations (Table 3.1). The pairwise comparisons of F_{ST} values for randomized *coastal* populations showed no population differentiation when two sets of 10 individuals were randomly drawn from within the same population, i.e. consisting of only *Coastal Shannon* (mean: -0.0005, 95% CI: -0.0086 – 0.0080) or *Coastal mobile* (mean: 0.0021, 95% CI: -0.0074 – 0.0115) individuals (Fig. 3.8). However, significant population differentiation was observed in comparisons of 10 individuals randomly drawn from one population with 10 individuals randomly drawn from the other (mean F_{ST}: 0.1820, 95% CI: 0.1589 – 0.2051) indicating a true population differentiation that was not driven by the sampling of closely related individuals or uneven sampling. The simulations run in POWSIM 4.1 (Ryman & Palm 2006) indicated that the power to detect a differentiation of $F_{ST} \geq 0.1$ between the two coastal populations was >0.99 with the set of 15 microsatellite markers used in the present study, even with a low sample size of 10 individuals drawn from each population.

Table 3.1 Pairwise F_{ST} and Jost's *D* values based on 15 microsatellite loci (given as average with 95% HPDI) between the different populations identified by STRUCTURE: *Coastal Shannon*, *Coastal mobile* and *Pelagic*. Values above the diagonal are for the whole dataset, and values below the diagonal after removal of close relatives ($r \geq 0.45$).

F_{ST}			
	Coastal Shannon	*Pelagic*	*Coastal mobile*
Coastal Shannon	-	0.173 (0.151-0.200)	0.181 (0.147-0.218)
Pelagic	0.154 (0.131-0.181)	-	0.186 (0.154-0.222)
Coastal mobile	0.161 (0.121-0.205)	0.172 (0.139-0.209)	-

Jost's *D*			
	Coastal Shannon	*Pelagic*	*Coastal mobile*
Coastal Shannon	-	0.362 (0.304-0.426)	0.207 (0.165-0.251)
Pelagic	0.339 (0.279-0.404)	-	0.319 (0.265-0.378)
Coastal mobile	0.188 (0.137-0.244)	0.305 (0.250-0.369)	-

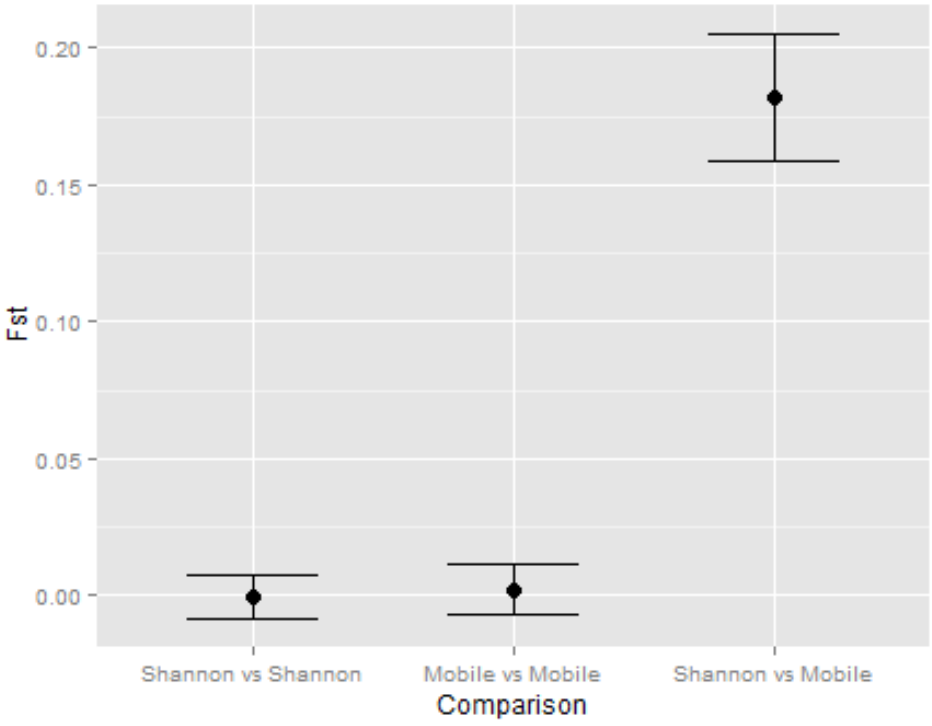

Figure 3.8 Average F_{ST} values (with 95% CI) from ten runs with 20 randomly selected individuals from either *Coastal Shannon*, *Coastal mobile* or from both populations after dividing individuals randomly into two populations of ten.

Sex-biased dispersal and migration rates

No evidence of sex-biased dispersal was found in any of the indices used (Table 3.2). The inferred migration rates (the proportion of migrants per population) calculated with BAYESASS were generally low (Table 3.3; see also Appendix 3.6); highest rates were found from *Pelagic* to *Coastal mobile* (mean: 0.036, 95% CI: -0.014 – 0.086) and from *Coastal mobile* to *Coastal Shannon* population (mean: 0.034, 95% CI: -0.011 – 0.078), and lowest from *Coastal Shannon* to *Coastal mobile* (mean: 0.008, 95% CI: -0.007 – 0.022) and to *Pelagic* (mean: 0.006, 95% CI: -0.005 – 0.017) (Table 3.3). Nevertheless, the migration rates between any of the putative populations were non-significant as zero was included in the range of 95% confidence intervals in each comparison.

When looking at individual posterior probabilities of migrant ancestry, two individuals from the *Coastal mobile* population and one from the *Pelagic* population had >50% probability of being either 1st or 2nd generation migrants from other populations. Two

Table 3.2 Indices used when testing for sex-biased dispersal and their corresponding *P*-values. The tests were run with the three populations identified with STRUCTURE.

	N	mAIc	vAIc	F_{IS}	F_{ST}
Females	31	-1.237	14.549	-	-
Males	66	0.618	11.880	-	-
Test statistic		-2.318	1.225	-0.017	-0.030
P-value		0.987	0.288	0.773	0.625

mAIc and vAIc = mean and variance of log-transformed corrected assignment indices, respectively.

Table 3.3 Inferred (posterior) mean migration rates (with 95% HPDI) between the different Irish bottlenose dolphin populations identified by STRUCTURE and DAPC, given as proportion of migrants per population. Values for self-recruitment are given in diagonal.

		Sink		
		Coastal Shannon	*Pelagic*	*Coastal mobile*
Source	*Coastal Shannon*	0.987 (0.969-1.000)	0.006 (-0.005-0.017)	0.008 (-0.007-0.022)
	Pelagic	0.016 (-0.014-0.046)	0.948 (0.892-1.000)	0.036 (-0.014-0.086)
	Coastal mobile	0.034 (-0.011-0.078)	0.012 (-0.010-0.034)	0.955 (0.906-1.000)

individuals from the *Coastal mobile* population ('tt-09-12' and '12-09-2014_Tt2') were 2nd generation migrants from the *Coastal Shannon* population with 64% and 79% probability, respectively. One individual assigned to the *Pelagic* population by STRUCTURE ('bnd204') had a 37% probability of being a 1st generation migrant and a 46% probability of being a 2nd generation migrant from the *Coastal mobile* population. When the individual that was biopsied in the Shannon Estuary but genetically assigned to *Coastal mobile* population ('tt-05-03') was grouped together with other Shannon individuals, it had a 19% probability of being a 1st generation migrant and a 70% probability of being a 2nd generation migrant from the *Coastal mobile* population.

Social structure and site fidelity

When testing for preferred and avoided companionships between and within the two coastal populations, the mean HWI in the real data was found to be significantly higher compared to the HWI of a permuted random data set (mean: $P < 0.01$, SD: $P < 0.0001$ and CV: $P < 0.0001$) indicating significant preferred short- and long-term companions. Moreover, the proportion of non-zero elements was larger in the random data compared to real data which suggests that some individuals may avoid others (Whitehead 2009), both within each population and between the two coastal populations (Fig. 3.9). The latter comes as no surprise since the two populations have not been documented associating with each other. Pairwise associations within the *Coastal Shannon* population were best described by the SLAR-model 'casual acquaintances', by which dyads remain associated for a period of time, dissociate and may or may not re-associate (Whitehead *et al.* 1991; Whitehead 2015). Within the *Coastal mobile* population, on the other hand, the model 'constant companions and casual acquaintances' best explained the data, with 'constant companions' remaining associated with each other throughout the length of the study (Whitehead *et al.* 1991; Whitehead 2015) (Fig 3.10). The mean HWI within the *Coastal Shannon* was 0.08 (SD = 0.09) and within the *Coastal mobile* population it was 0.23 (SD = 0.21).

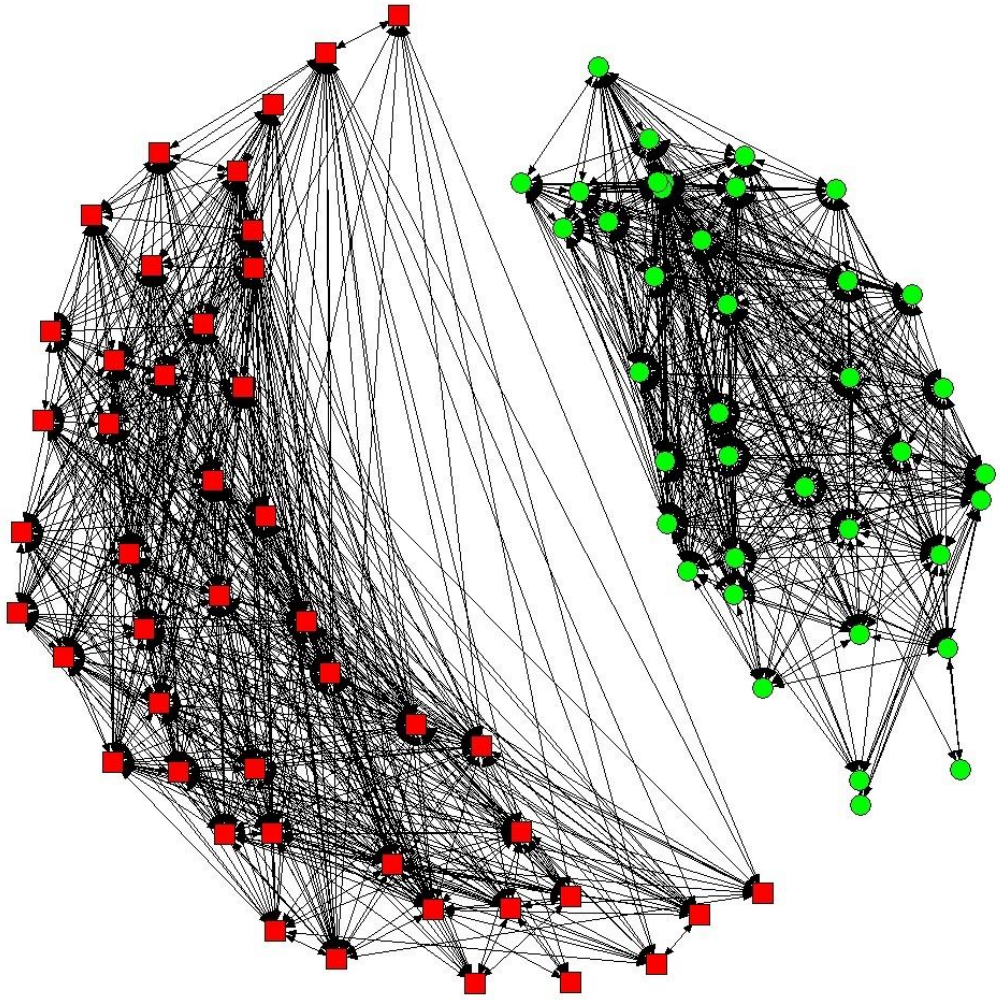

Figure 3.9 Social network diagram of bottlenose dolphins encountered at least on five occasions during the data collection 1996-2014. Boxes represent individuals encountered in the Shannon estuary, and circles the 'mobile' dolphins encountered on the west and north-west coast of Ireland. The length of the line in the network diagram inversely represents the strength of the association between a dyad calculated as HWI.

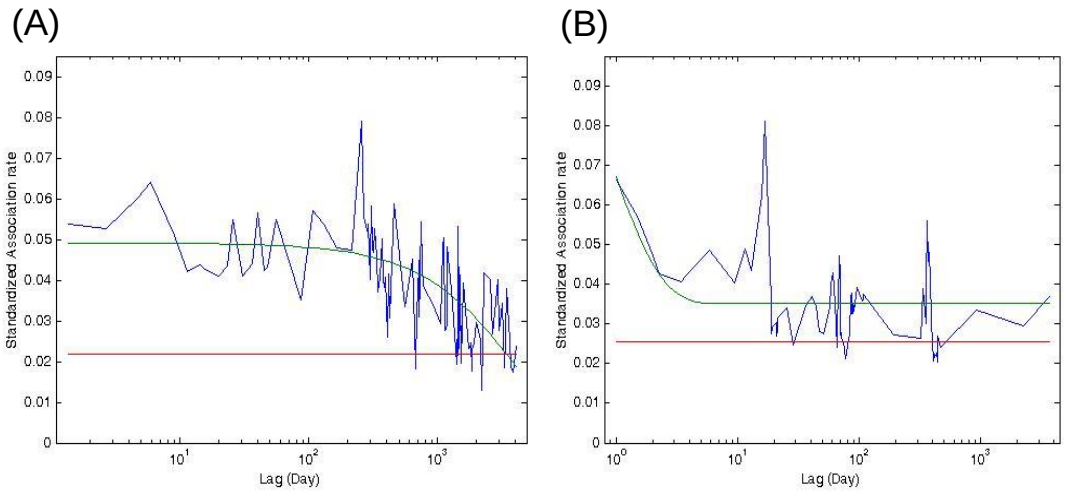

Figure 3.10 Standardized Lagged Association Rate (SLAR), the probability that associates remain together divided by the mean number of associates, for bottlenose dolphins encountered ≥5 times (A) in the Shannon Estuary, and (B) outside Shannon Estuary in the coastal waters of Ireland, during the study period 1996-2014. SLAR is represented by the blue line, null association rate (expected if all individuals associate at random) by the red line, and the best fitting model by the green line ("casual acquaintances" in the *Coastal Shannon*, and "constant companions and casual acquaintances" in the *Coastal mobile* subset). Time lag (number of days) is given on logarithmic scale.

Bottlenose dolphins that were first photographed in the Shannon Estuary were not photographed anywhere else during 1996-2008 except once in Brandon Bay, Co. Kerry (approximately 30km south from the mouth of the Shannon Estuary), hence their annual average LIR was zero to any other study area, except to Brandon Bay where it was 0.0263 (SE = 0.0128). Likewise, dolphins belonging to the *Coastal mobile* population were never photographed in the Shannon Estuary during the study period so their LIR in the Shannon Estuary was also zero. The LIR within the Shannon stayed fairly constant for approximately 100 days, followed by some fluctuations in the rate (Fig. 3.11a). Two competing models had substantial support explaining the data, with the emigration/mortality model having the lowest AIC value, followed by emigration+reimmigration+mortality model (Table 3.4). LIR associated with the *Coastal mobile* population was best explained by the emigration+reimmigration+mortality model (Table 3.4; Fig. 3.11b).

Table 3.4 Lagged Identification Rate (LIR) models (with their respective AIC/QAIC values) fitted to explain the probability to encounter individual bottlenose dolphins that had been encountered before within the Shannon estuary (*Coastal Shannon*) or outside of it (*Coastal mobile*).

Area	Model	Explanation	AIC/QAIC
Coastal Shannon	(a1)	Closed	74504.7
	(a2*exp(-a1*td))	Emigration/mortality	**74173.2**
	(a2+a3*exp(-a1*td))	Emigration+reimmigration	74502.2
	(a3*exp(-a1*td)+a4*exp(-a2*td))	Emigration+reimmigration+ mortality	74177.0
	(a1*cos(a2*td)+a3)	Seasonal emigration+reimmigration	74508.7
Coastal mobile	(a1)	Closed	4190.4*
	(a2*exp(-a1*td))	Emigration/mortality	**4163.0***
	(a2+a3*exp(-a1*td))	Closed: Emigration+reimmigration	4177.0*
	(a3*exp(-a1*td)+a4*exp(-a2*td))	Emigration+reimmigration +mortality	4168.3*
	(a1*cos(a2*td)+a3)	Seasonal emigration+reimmigration	4193.9*

a1 = emigration rate, a2 = mean time in study area, a3 = mean time out of study area, a4 = Mortality rate, td = time lag, *N* = population size in the study area. * denotes QAIC-values with variation inflation factor of 2.1.

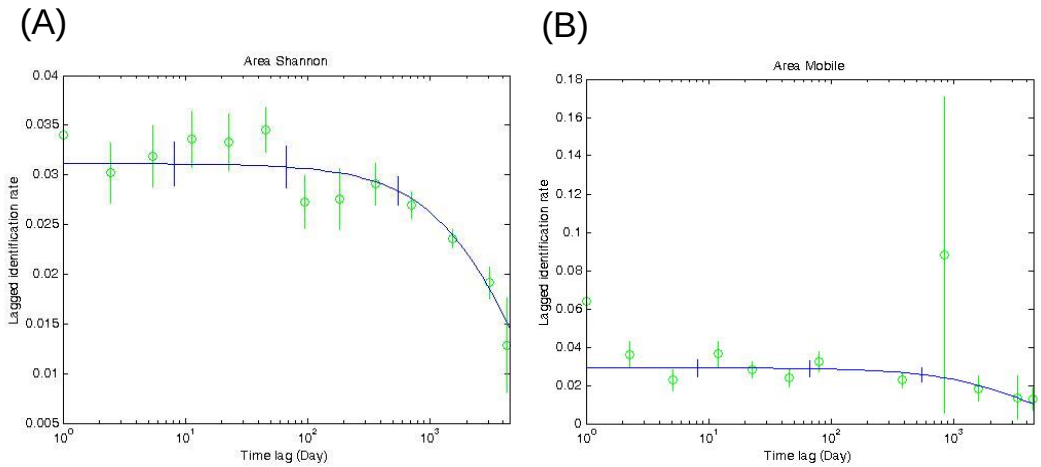

Figure 3.11 Lagged identification rate (LIR) for bottlenose dolphins encountered ≥5 times (A) in the Shannon Estuary, and (B) outside the Shannon Estuary in the coastal waters of Ireland during the study period 1996-2014. The graph describes the probability that a dolphin photographed at time 0 will be identified again at time X within the area. Data points are represented as green circles (with SE) and the best fitting model ("emigration/mortality" in the *Coastal Shannon*, and "emigration+reimmigration+mortality" in the *Coastal mobile* subset) is displayed as the blue line. Time lag (number of days) is given on logarithmic scale.

Relatedness, spatial overlap and associations

When only the biopsied individuals with sufficient number of photo-ID encounters (≥3) were considered, a significant correlation was found between the relatedness coefficient (Queller & Goodnight 1989) and HWI (r = 0.345, P = 0.0001) when the data from the two coastal populations were combined. However, this was likely attributed to the correlation of zero values in the combined data set since no correlation was found between the two indices when testing for this separately for each population (*Coastal Shannon* r = 0.028, P = 0.363; *Coastal mobile* r = 0.0004, P = 0.480). Out of fifteen dyads with significant associations (P < 0.05), none had $r \geq 0.45$, but three dyads had r-values close to 0.25 indicating possible half-siblings or cousins. No correlation was found between relatedness and spatial overlap within the *Coastal Shannon* (r = 0.076, P = 0.193) or the *Coastal mobile* population (r = 0.042, P = 0.417). Overall, these results indicate that close kinship may not strongly promote overall social associations in these two populations.

3.4 Discussion

Understanding the scale of dispersal is an important consideration for the conservation and management of marine species (Lotterhos 2012). By combining genetic and photo-identification data both spatial and genetic dispersal over both short and long temporal scales have been elucidated in unprecedented detail for bottlenose dolphins in Irish waters. Dispersal can be gametic, *i.e*. via gene flow during temporary interactions and spatial overlap, and therefore only detected by genetic methods. Dispersal can also be demographic, *i.e.* the permanent movement of individuals from one location to another, detectable over the short-term using photo-identification of naturally marked individuals and over the past few generations using genetic methods (relatedness, migration and admixture proportions; Iacchei *et al.* 2013). The combined results indicate social and reproductive isolation between the three identified populations, with only low levels of demographic and potential genetic connectivity *sensu* Lowe and Allendorf (2010). The accumulation of differentiation, estimated as F_{ST}, indicates that this relative isolation has persisted over longer timescales.

Among the bottlenose dolphin samples, large and significant F_{ST} and Jost's *D* values between the populations, comparison of F_{ST} values from randomized 'coastal populations', the individual assignment methods and kinship methods were all in agreement supporting the division of the samples into one '*pelagic'* and two *'coastal'* clusters. In addition, Jost's *D* values and DAPC indicated a presence of a hierarchical population structure with the largest genetic difference occurring between the '*pelagic*' and '*coastal*' populations. Furthermore, social structure analyses using long-term photo-identification data revealed that the two coastal populations were not only genetically, but also socially distinct. This kind of social separation has been previously reported between the '*pelagic*' and '*coastal*' bottlenose dolphins (Oudejans *et al.* 2015).

The results also suggest that both coastal populations show a similar degree of site fidelity to their respective areas and are likely to have non-overlapping core home ranges, at least during the seasons that photo-id work has been conducted. The gradual decrease in the Lagged Identification Rates (LIRs) towards the end of the study period reflects a decrease in site-fidelity that is likely explained by mortality and/or emigration. These results highlight that a high degree of site-fidelity, especially

evident in the Shannon Estuary SAC where data have been collected for over 12 years, is a key driver of fine-scale population structure among coastal populations. A high degree of site-fidelity among resident populations of bottlenose dolphins to certain local areas has been found in other parts of the world (Simoes-Lopez & Fabian 1999; Bristow & Rees 2001; Möller *et al.* 2002). This residency, found especially in embayments, coupled with genetic differentiation between dolphins residing in adjacent coastal habitats, has led a number of authors to suggest that variability in these habitats and the ability of local populations to accommodate it by the development of different foraging strategies (e.g. Smolker *et al.* 1997; Barros & Wells 1998) may have shaped the fine-scale population structure among these dolphins (Hoelzel *et al.* 1998; Chilvers & Corkeron 2001 Natoli *et al.* 2005; Möller *et al.* 2007; Sargeant *et al.* 2007; Richards *et al.* 2013; Allen *et al.* 2016). However, this is yet to be tested using adaptive genetic markers, such as certain Single Nuclear Polymorphisms (SNPs). In addition, there is growing evidence that cultural transmission occurs within dolphin social communities (e.g. Krützen *et al.* 2005; Mann *et al.* 2012) in the form of social learning which may facilitate the evolution of specialist foraging behaviours, which in turn has the potential to maintain population structure between adjacent communities (isolation by adaptation). In this study, there is evidence of short and/or long-term preferred companionships within the two coastal populations, and it is possible that social bonds promote and maintain the observed social and genetic separation of these populations. Perhaps surprisingly, the companionships did not seem to be linked to relatedness but close associates were found among kin and non-kin individuals. In contrast, close associations were linked to relatedness among females in a population of Indo-Pacific bottlenose dolphins (Möller *et al.* 2006), and support for relatedness in male groups has been documented in alliances of this genus (Krützen *et al.* 2003) as well as among short-beaked common dolphins (*Dephinus delphis*) in southern Australia with greater relatedness found between males within schools than between schools (Zanardo *et al.* 2016). Unfortunately, there were insufficient combined photo-ID and genetic data to investigate possible sex-specific patterns in the relatedness and associations among the two coastal Irish populations partly due to genetic sampling being biased towards males given their more distinct markings (especially in the *Coastal Shannon* population) and partly because of the fact that the biopsy sampled animals did not necessarily have enough photo-ID encounters for further social analyses.

Lowe and Allendorf (2010) described genetic connectivity as the exchange of alleles through gene flow between populations, and demographic connectivity as the dispersal of individuals from one population to another thus contributing to underlying population demographic processes and parameters (e.g. survival, mortality, abundance). Gene flow maintains genetic variation in populations, enhancing adaptive potential to respond to environmental variation (Yamamichi & Innan 2012). Even small amounts of gene flow can prevent the accumulation of large genetic differences between populations of low effective size (Slatkin 1987; Palumbi 2003). Hastings (1993), on the other hand, suggested that populations become demographically isolated if the exchange between populations stays below 10%, i.e. less than 10% of the population growth is contributed by migrants from other populations regardless of whether they contribute to the gene flow or not. Palsbøll *et al.* (2006) further recommended that in such cases, populations should be considered as separate MUs. Recent migration rates between the different Irish bottlenose dolphin populations were non-significant (*i.e.* zero) in all comparisons inferred using BAYESASS. However, one individual ('tt05-03') encountered over nine years in the Shannon Estuary, was genetically assigned to the *Coastal mobile* population. Interestingly, this dolphin has never been photographed associating with the *Coastal mobile* population, but no close kin were found among the genotyped individuals assigned to the *Coastal Shannon* population. Given that ~40% of the *Coastal Shannon* population have been biopsied, it is possible that this dolphin has not genetically contributed to the *Coastal Shannon* population. However, close kinship was found between 'tt05-03' and an individual sampled within the *Coastal mobile* population. Thus, 'tt05-03' appears to be an example of demographic dispersal from the *Coastal mobile* population to the *Coastal Shannon* population. However, there is yet no evidence that this dispersal event has led to the dispersal of gametes between the two coastal populations.

No evidence for sex-biased dispersal was found in this study; however, the sampling was biased towards males with more than double the amount of samples compared to females, thus these results should be treated with caution. Both Mirimin *et al.* (2011) and Louis *et al.* (2014a) found two haplotypes that were shared between 'coastal' and 'pelagic' dolphins, but the sequencing of the entire mitochondrial genome (Chapter 2) revealed no shared haplotypes between individuals sampled from either of the coastal populations and pelagic population suggesting limited female dispersal between

coastal and pelagic populations. However, two identical mitogenomes (*i.e.* haplotypes) were shared between the two coastal populations occurring in Irish waters, suggesting either that some movement between these populations exists via female mediated gene flow, or that the shared haplotypes are a consequence of recent divergence between the two populations.

Two individuals strongly assigned to the *Coastal mobile* population with assignment methods were identified as likely 2nd generation migrants originating from the *Coastal Shannon* population with BAYESASS. However, whilst individual assignment methods, such as STRUCTURE, are believed to perform well at identifying migrant individuals (Putman and Carbone 2014), BAYEASS was found to be less reliable in calculating individual migrant probabilities (Faubet *et al*. 2007); thus these results should be interpreted with caution. Nevetheless, BAYEASS was found to perform well at estimating overall migration rates between populations over a few generations at migration rates up to 0.1 (Faubet *et al*. 2007). Whether there is more dispersal between the two coastal populations is uncertain and warrants more sampling effort especially within the *Coastal mobile* population. To date, only ~12% of this population occurring in Irish waters has been sampled, based on a median abundance estimate of 189 dolphins (derived from mark-recapture estimates using photo-ID data, see Chapter 4). Overall, despite some evidence for low levels of demographic dispersal it appears that connectivity between populations is too low to prevent the build-up of genetic differentiation.

Nichols *et al.* (2007) and Louis *et al.* (2014a) suggested that coastal bottlenose dolphins in northern European waters may form a wider meta-population (the '*Coastal North*' meta-population, Louis *et al.* 2014a) consisting of inter-connected local populations around the British Isles. However, these studies did not include samples from the *Coastal Shannon* population, which is, based on this study, both genetically and demographically isolated. Coupled with the relatively small effective population size this makes *Coastal Shannon* especially vulnerable to any environmental or anthropogenic stressors. The *Coastal mobile* population, on the other hand, may belong to this '*Coastal North*' meta-population. The full extent of the ranges of individuals in any of the populations studied here is not known, but previous research has shown that at least some of these mobile coastal animals travel over distances at

the scale of hundreds of kilometers (Ingram *et al.* 2001, 2003; O'Brien *et al.* 2009; Robinson *et al.* 2012, Cheney *et al.* 2013). If they do indeed comprise part of the '*Coastal North*' meta-population extending beyond Irish waters, trans-national co-operation, monitoring and management may be needed. Six individuals from the west coast of Ireland have been matched on an *ad-hoc* basis to photo-ID catalogues comprised of animals ranging in the coastal waters of Scotland (Robinson *et al.* 2012) but there is a need for a collaborative effort and consistent scientific approach to better integrate photo-ID catalogues from different regions/countries (e.g. Ireland, Wales, Scotland, France, Cornwall). Such collaboration would provide better insights into demographic dispersal, ranging patterns and the abundance of this putative meta-population. In addition, genetic dispersal within the meta-population needs to be quantified through increased sampling effort over a larger area extending beyond country boundaries and using a common set of genetic markers that are comparable between laboratories. To this end, a recent SNP discovery study has developed genome-wide markers (see Appendix 6.1, Chapter 6: Louis, Nykänen *et al.* in prep.).

The present study supports the delineation of the three populations occurring in Irish waters as separate management units based on the low genetic, social and demographic dispersal between the populations thus validating the current designation of separate SACs for the two coastal populations. The study also highlights the importance of distinguishing genetic and demographic connectivity so that gene flow can be differentiated from dispersal that has no subsequent genetic contribution from the migrant to the local population. The quantification of migration rates and the degree of social connectivity, on the other hand, have implications in the delineation of MUs, especially in cases where population structuring is not clear. With this information the functioning of existing marine protected areas or networks can be better assessed and the need for designating new protected areas evaluated.

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Chapter 3: Appendices

Appendix 3.1 Basic genetic indices for 15 microsatellite loci in the three populations identified by Structure and DAPC, *Coastal Shannon* (*N* = 54, including individuals sampled in Cork harbour), *Coastal mobile* (*N* = 25) and *Pelagic* (*N* = 18).

	Coastal Shannon									
	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} Low	F_{IS} High
D18[a]	54	3	33.3	2.2	0.150	0.140	1.000	-0.071	-0.124	-0.027
D22[a]	51	4	40.0	3.7	0.570	0.620	0.715	0.083	-0.109	0.281
*Dde*59[b]	52	4	50.0	4.0	0.790	0.710	0.893	-0.116	-0.261	0.040
*Dde*61[b]	52	3	60.0	3.0	0.690	0.580	0.096	-0.187	-0.350	-0.016
*Dde*65[b]	51	4	66.7	3.6	0.530	0.450	0.108	-0.167	-0.277	-0.043
*Dde*66[b]	50	2	20.0	2.0	0.200	0.180	1.000	-0.111	-0.185	-0.050
*Dde*69[b]	54	4	80.0	4.0	0.610	0.540	0.810	-0.136	-0.264	0.016
*Dde*72[b]	53	5	55.6	4.8	0.570	0.600	0.660	0.056	-0.103	0.225
GATA098[c]	52	4	44.4	4.0	0.730	0.700	0.468	-0.037	-0.182	0.119
*Ttr*04[d]	54	3	37.5	3.0	0.700	0.620	0.654	-0.141	-0.318	0.046
*Ttr*11[d]	53	3	30.0	3.0	0.640	0.580	0.637	-0.113	-0.293	0.079
*Ttr*34[d]	53	4	66.7	3.2	0.640	0.550	0.134	-0.174	-0.402	0.056
*Ttr*48[d]	54	4	57.1	4.0	0.700	0.640	0.875	-0.093	-0.242	0.068
*Ttr*63[d]	54	9	52.9	7.6	0.910	0.850	0.784	-0.070	-0.157	0.030
*Ttru*AAT44[e]	54	4	50.0	4.0	0.590	0.580	0.241	-0.023	-0.198	0.163
Overall	53	60	49.6	3.7	0.600	0.560	0.847	-0.083	-0.130	-0.038*

	Coastal mobile									
	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} Low	F_{IS} High
D18[a]	25	4	44.4	3.4	0.520	0.580	0.273	0.108	-0.243	0.457
D22[a]	24	4	40.0	3.5	0.500	0.560	0.198	0.101	-0.259	0.468
*Dde*59[b]	25	3	37.5	2.8	0.520	0.540	0.604	0.031	-0.321	0.389
*Dde*61[b]	25	3	60.0	2.9	0.280	0.390	0.089	0.283	-0.092	0.657
*Dde*65[b]	25	2	33.3	2.0	0.320	0.320	1.000	0.000	-0.296	0.424
*Dde*66[b]	25	2	20.0	1.5	0.000	0.080	0.020	1.000	1.000	1.000
*Dde*69[b]	24	3	60.0	3.0	0.580	0.530	1.000	-0.098	-0.338	0.178
*Dde*72[b]	25	4	44.4	3.8	0.480	0.570	0.029	0.152	-0.141	0.464
GATA098[c]	24	4	44.4	3.8	0.750	0.660	0.541	-0.131	-0.340	0.095
*Ttr*04[d]	25	4	50.0	4.0	0.640	0.630	0.716	-0.015	-0.268	0.255
*Ttr*11[d]	24	3	30.0	2.8	0.500	0.390	0.725	-0.274	-0.445	-0.138
*Ttr*34[d]	25	5	83.3	5.0	0.800	0.760	0.848	-0.046	-0.226	0.152
*Ttr*48[d]	25	3	42.9	2.9	0.280	0.250	1.000	-0.118	-0.214	-0.041
*Ttr*63[d]	24	6	35.3	5.3	0.710	0.610	0.883	-0.154	-0.303	0.008
*Ttru*AAT44[e]	25	4	50.0	3.8	0.520	0.630	0.291	0.170	-0.093	0.450
Overall	25	54	45.0	3.4	0.490	0.500	0.361	0.013	-0.066	0.088

	Pelagic									
	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} Low	F_{IS} High
D18[a]	18	8	88.9	7.5	0.890	0.840	0.827	-0.055	-0.193	0.120
D22[a]	18	9	90.0	8.2	0.890	0.830	0.354	-0.067	-0.220	0.115
*Dde*59[b]	18	8	100.0	7.1	0.720	0.820	0.294	0.120	-0.156	0.413
*Dde*61[b]	18	5	100.0	4.9	0.720	0.730	0.039	0.013	-0.269	0.299
*Dde*65[b]	18	5	83.3	4.7	0.890	0.750	0.856	-0.180	-0.336	0.012
*Dde*66[b]	18	10	100.0	7.7	0.720	0.680	0.281	-0.064	-0.269	0.129
*Dde*69[b]	17	5	100.0	4.9	0.820	0.750	0.476	-0.104	-0.352	0.157
*Dde*72[b]	18	8	88.9	7.8	0.940	0.850	1.000	-0.105	-0.214	0.033
GATA098[c]	18	8	88.9	7.0	0.830	0.770	0.362	-0.084	-0.294	0.136
*Ttr*04[d]	18	8	100.0	7.4	0.830	0.830	0.346	0.000	-0.187	0.201
*Ttr*11[d]	18	10	100.0	8.4	0.890	0.820	0.881	-0.079	-0.215	0.076
*Ttr*34[d]	17	6	100.0	5.5	0.710	0.760	0.843	0.077	-0.174	0.351
*Ttr*48[d]	17	7	100.0	6.5	0.820	0.810	0.136	-0.022	-0.224	0.201
*Ttr*63[d]	18	14	82.4	10.6	0.830	0.830	0.567	-0.002	-0.167	0.188
*Ttru*AAT44[e]	18	8	100.0	6.9	0.830	0.780	0.353	-0.065	-0.251	0.146
Overall	18	119	94.8	7.0	0.820	0.790	0.585	-0.040	-0.096	0.013

N = number of individuals, A = number of alleles observed, % = percentage of total alleles, AR = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, all are per locus per population sample. HWE = corrected *P*-values (chi-square test for goodness-of-fit), F_{IS} = F_{IS} values for each loci and population sample (overall), F_{IS} Low/High = bias corrected 95% confidence interval. *denotes significance in F_{IS} values over all loci.

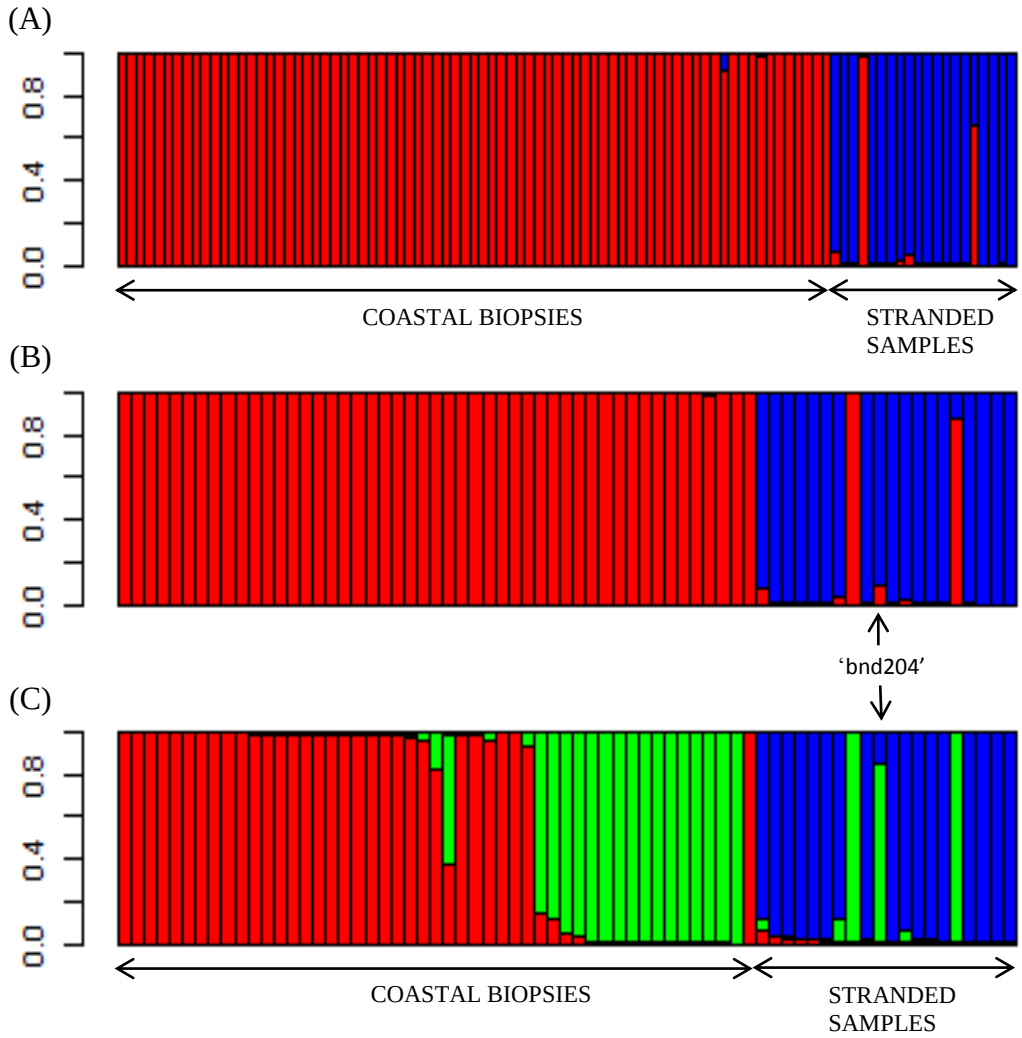
[a]Shinohara *et al.* 1997.

[b]Coughlan *et al.* 2006.

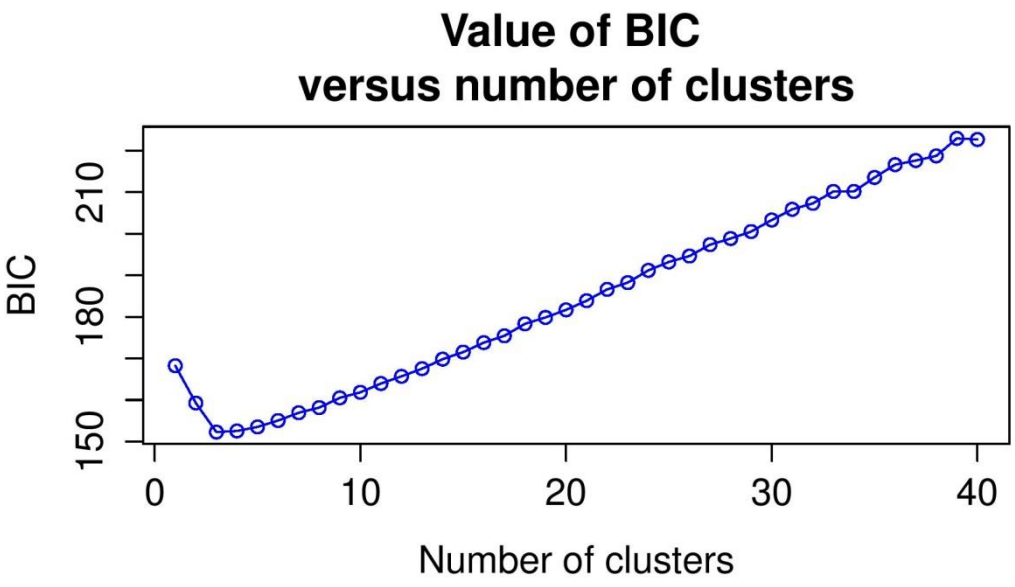
[c]Palsbøll *et al.* 1997.

[d]Rosel *et al.* 2005.

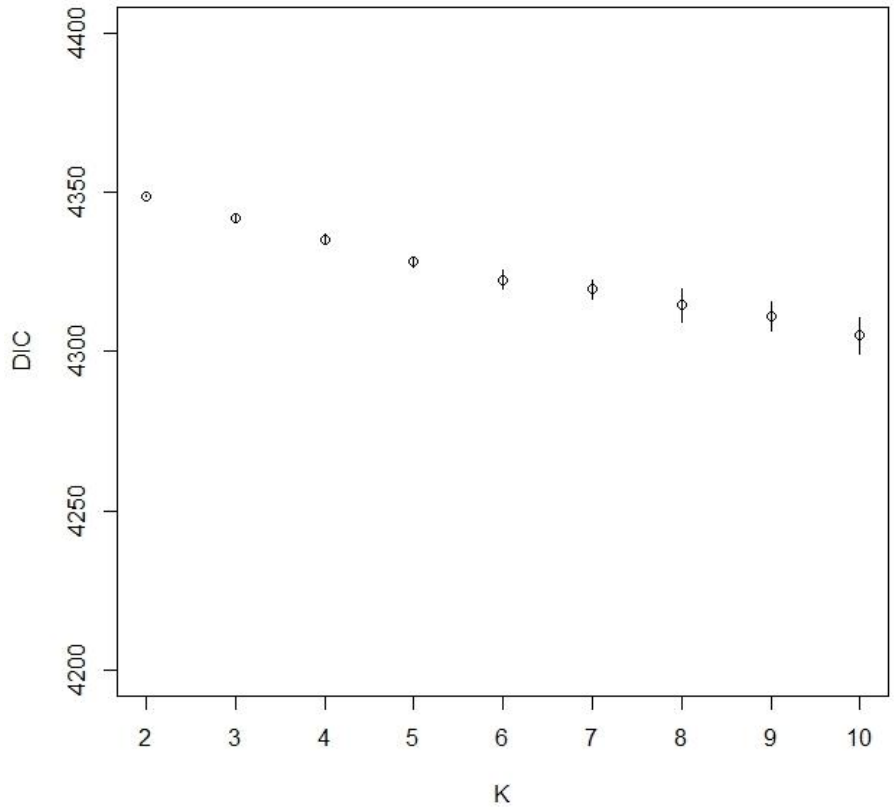
[e]Caldwell *et al.* 2002.



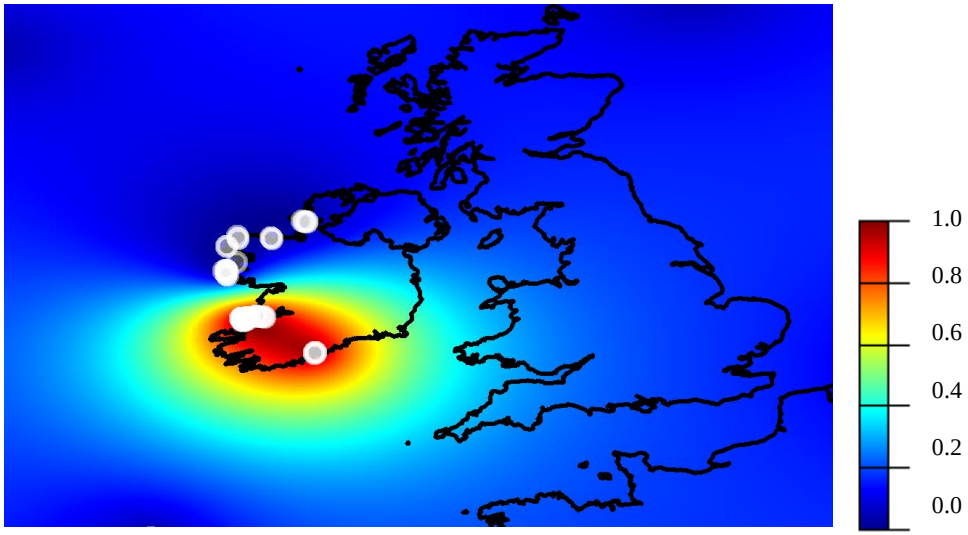
Appendix 3.2 Population assignment probabilities (STRUCTURE) of (A) all Irish bottlenose dolphins with $K = 2$, (B) after removal of close relatives ($r \geq 0.45$) from the dataset with $K = 2$, and (C) after removal of close relatives ($r \geq 0.45$) from the dataset with $K = 3$, where K is the candidate number of populations. Each vertical column corresponds to an individual dolphin. Note that sample 'bnd204' is assigned to 'pelagic' cluster at $K = 2$ (B) and to 'coastal mobile' cluster at $K = 3$ (C), when close relatives are removed.



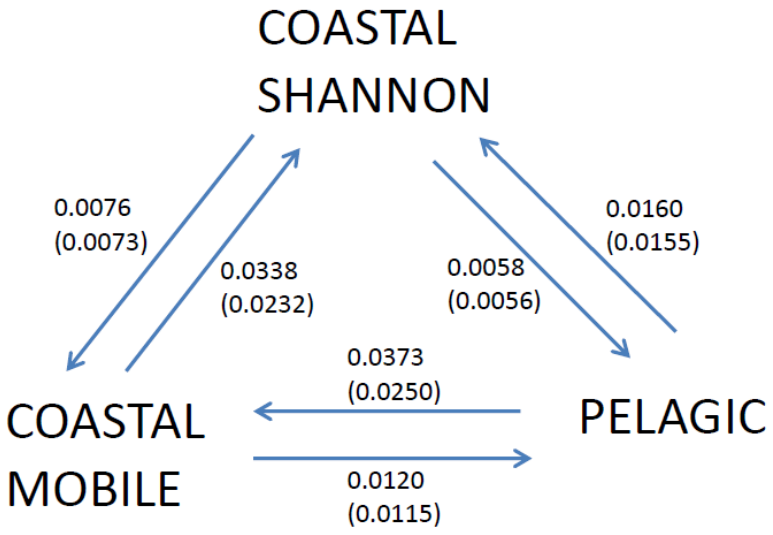
Appendix 3.3 Bayesian Information Criterion (BIC) values plotted against the number of clusters. The lowest BIC-value indicates the most likely number of clusters (K = 3).



Appendix 3.4 Mean Deviance Information Criterion (DIC) values (with SD) using ten replicate TESS runs for each candidate number of populations (K) varying from 2 to 10.



Appendix 3.5 Map of individual assignment probabilities (TESS) of bottlenose dolphins belonging to *Coastal Shannon* population, including biopsy samples from Cork harbour. White dots are biopsy locations and the colour scale bar indicates the assignment probabilities.



Appendix 3.6 Inferred (posterior) mean migration rates (with SD) expressed as the number of migrants per generation between the different Irish bottlenose dolphin populations identified by STRUCTURE and DAPC.

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Chapter 4: Using Bayesian inference with a multi-site mark-recapture model to estimate the abundance of a mobile population of bottlenose dolphins, *Tursiops truncatus*, on the west coast of Ireland

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Abstract

Past research effort on bottlenose dolphins in Ireland has largely concentrated on a semi-resident population occupying the Shannon Estuary and the associated Lower River Shannon SAC designated for this population, but less is known about a coastal population using multiple sites throughout the Irish west coast. Especially in light of the MSFD and the recent designation of a separate SAC (West Connacht Coast SAC) for this transient coastal population, it is essential to set up a monitoring strategy for these animals. In this study, mark-recapture methods were applied to Bayesian inference and hierarchical log-linear likelihood to derive a multi-site abundance estimate of coastal bottlenose dolphins for the wider Connemara-Mayo-Donegal area in 2013 and 2014. Well-marked individuals photographed during dedicated boat-based surveys at different sites on the Irish west coast were identified from high-quality photographs taken May–August 2013 and June–September 2014 and included in the analysis. The model-averaged median estimate for the abundance of well-marked and unmarked dolphins in the whole study area in 2013 was 145 (CV = 0.30, 95% HPDI = 111–239), and in 2014 it was 189 (CV = 0.11, 95% HPDI = 162–232). High rates of movement between the different areas were observed during the study. The dolphins used the entire study area during the two years of survey effort, with nearly half (43%) of all well-marked identified animals sighted in more than one of the three survey blocks during 2013–2014. Given that the SAC designated for these animals covers a substantial area of the west coast of Ireland, the Bayesian multi-site approach is appropriate and can be applied for monitoring this population. It is well-suited for sparse recapture data collected opportunistically at multiple sites when systematic line-transect surveys are often unfeasible due to changeable weather conditions and unpredictable occurrence of the animals. With a mobile population with such extensive movements such as this one, monitoring the abundance on the dedicated SAC alone would likely lead to underestimation of the true numbers of this population.

4.1. Introduction

The size and distribution of animal populations can change in time due to variety of reasons. Continuous assessment of the status of populations and determining the reasons for these changes are key concepts of conservation research in the attempts towards preserving biodiversity. The identification of management units (MUs), usually defined as demographically independent populations whose growth rate depend almost solely on intrinsic birth and death rates rather than immigration (Palsbøll *et al.* 2006) is the initial step towards successful conservation and management strategies. Marine Protected Areas (MPAs) can then be designated for the species/populations to cover at least their core range, and subsequent monitoring of the abundance and others demographic parameters commenced. Obtaining accurate estimates of population size and determining trends in abundance due to demographic changes such as increased mortality or emigration should be an integral part of any management strategy allowing any changes such as a possible shift in distribution (*e.g.* Wilson *et al.* 2004, MacLeod *et al.* 2005) to be detected early allowing more time for appropriate management actions.

As a cosmopolitan species, bottlenose dolphins, *Tursiops truncatus*, are found in coastal inshore waters, in continental shelf regions and in open ocean environments (Wells & Scott 2002), and their minimum worldwide abundance estimate totals to about 600,000 individuals (Hammond *et al.* 2012) and their abundance in European waters is around 16,000 (Hammond *et al.* 2013). While bottlenose dolphin as a species is not considered to be globally endangered, some populations, especially the ones inhabiting coastal areas, are small and often genetically and/or geographically isolated (Natoli *et al.* 2005; Parsons *et al.* 2002, 2006; Baird *et al.* 2009; Rosel *et al.* 2009; Fernández *et al.* 2011; Mirimin *et al.* 2011; Ansmann *et al.* 2012; Martien *et al.* 2012; Caballero *et al.* 2012; Gaspari *et al.* 2015a, 2015b; Louis *et al.* 2014). This puts them at risk of losing heterozygosity due to genetic drift alone (Lacy 1987) and thus may affect their ability to cope with different environmental stressors. Moreover, occupying coastal habitats potentially exposes them to high levels and a wide range of human impact.

All cetaceans are listed in Annex IV of European Union's Habitats Directive necessitating 'strict protection' for such species. In addition, bottlenose dolphins are

listed in Annex II of the Habitats Directive; this means that the Member States are required to designate Special Areas of Conservation (SACs) as part of European strategy to maintain or restore a favourable conservation status for the species (Natura 2000). Previous research effort on bottlenose dolphins in Ireland has largely concentrated on animals inhabiting the large open estuary of the River Shannon (*e.g.* Berrow *et al.* 1996, 2012; Ingram & Rogan 2002, 2003; Englund *et al.* 2007, 2008; Foley *et al.* 2012), and this population has been designated a SAC covering a considerable area of the lower part of the estuary (i.e. Lower River Shannon SAC). This area has been monitored since mid-1990s with summer abundance estimates calculated using data collected during several summer seasons. Estimates of abundance for dolphins using the Shannon SAC range from 114 to approximately 140 (Berrow *et al.* 1996, 2012; Ingram & Rogan 2002, 2003; Englund *et al.* 2007, 2008).

However, recent studies suggest that the bottlenose dolphins using the coastal habitats along the west coast of Ireland belong to two small, genetically and socially distinct populations which are further distinguished from a larger offshore population (see Chapter 2; Mirimin *et al.* 2011; Oudejans *et al.* 2015). Compared to the semi-resident population inhabiting the Shannon Estuary, much less is known about this second coastal population. Preliminary studies identified a significant number of bottlenose dolphins using the waters off the west coast of Ireland, and multi-annual re-sightings of individuals indicated that these animals belonged to a discrete assemblage of animals that appeared to be highly mobile with encounters occurring throughout the west coast (Ingram *et al.* 2001, 2003). Consequently, a second SAC for bottlenose dolphins was designated in Connemara, Co. Galway and western County Mayo (West Connacht Coast SAC) to protect this 'coastal mobile' population. In 2009, an abundance estimate of 171 (95% CI = 100–294) was derived from dedicated survey data collected during surveys of north Connemara waters (Ingram *et al.* 2009). This estimate represented the first attempt to assess the number of animals using a site outside of the Lower River Shannon SAC. However, the surveys were restricted to a relatively small area in Connemara, and from previous photo-identification work it was apparent that the animals were ranging well beyond this area with matches of individuals as far apart as Co. Cork and Co. Donegal (Ingram & Rogan 2003). Such large range and unpredictable movements make monitoring this population and deriving robust estimates of abundance especially challenging. Therefore, the aim of

this study was to obtain a more precise estimate of the number of bottlenose dolphins occupying a wider area on the west coast of Ireland, including the West Connacht Coast SAC and extending to Donegal Bay, Co. Donegal. Here, a Bayesian method suitable for opportunistic data collected from multiple sites over a wide geographic area (Durban *et al.* 2005) is used to estimate the abundance of this mobile population. In addition to estimating abundance, the ranging behaviour of identified individuals belonging to this transient population is examined.

4.2. Materials and methods

Boat-based photo-identification surveys

Three regional survey areas around the west and north-west coasts of Ireland, where bottlenose dolphins are frequently reported, were selected as the focus of this study. These areas broadly represent the coastal waters of north Connemara, Co. Galway, areas around Mullet Peninsula, Co. Mayo, and northern parts of Donegal Bay, Co. Donegal (Fig. 4.1), spanning over 300km along the coast. Dedicated boat-based surveys from a 6.5m rigid-hull inflatable boat (RIB) were conducted within these coastal blocks during the summers of 2013 and 2014. These surveys on predetermined tracks were conducted in Beaufort sea-states ≤3 with suitable ambient light and swell conditions with the purpose covering as much coastal area as possible in a single day, and if the weather conditions deteriorated, the survey was abandoned. In addition, opportunistic surveys (responses to sightings from the public) were conducted with the same aim as with the dedicated surveys, to locate and photograph schools of bottlenose dolphins.

A bottlenose dolphin school was defined as "all dolphins within a 100m radius of each other" (Irvine *et al.* 1981) and hereafter 'encounters' refer to periods of data collection whilst with dolphin schools. When sighted, dolphins were approached slowly and carefully, minimising changes in vessel speed and direction in order to reduce disturbance to the animals. Schools of dolphins were approached from a course that was parallel and convergent to the heading of the dolphins. Best efforts were made to photograph the dorsal fins of all members of the school during each encounter. Identification photographs were taken using a digital SLR camera (Canon EOS 1DS

mark II and Nikon D7100, 70-200mm telephoto lens) as close to perpendicular to the animals' dorsal fin as possible and preferably within a distance of 20m.

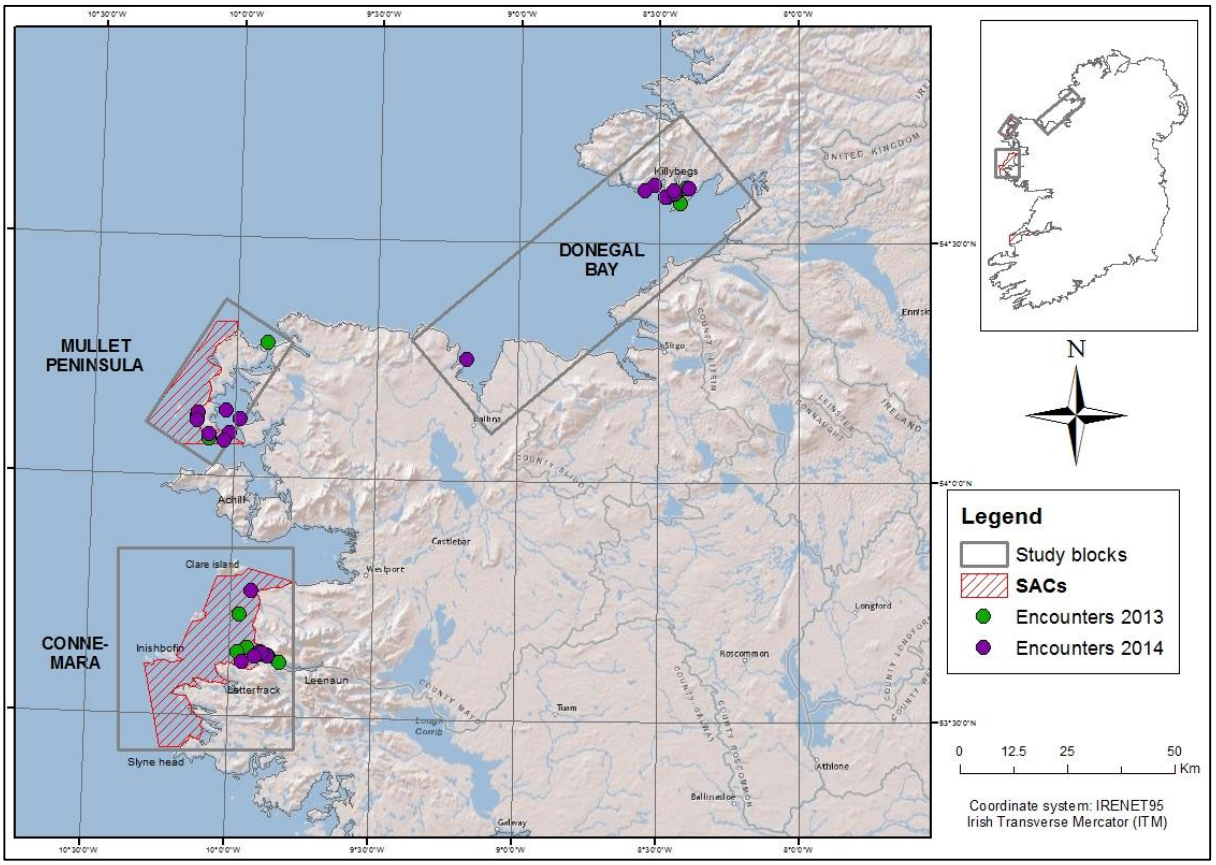

Figure 4.1 The three coastal 'blocks' surveyed during 2013-2014 and the West Connacht Coast Special Area of Conservation (SACs) designated to protect this population of bottlenose dolphins. Dots denote the location of encounters with bottlenose dolphin schools during the study period.

Photograph analysis

Individual bottlenose dolphins can be identified using their natural markings (Würsig & Würsig 1977; Würsig & Jefferson 1990). These marks mostly consist of scars and nicks from interactions with conspecifics and they can be permanent, such as deep nicks or scars on the dorsal fin, or temporary, such as superficial scratches (Fig. 4.2). Other types or marks include distinctive fin shape, body deformities and lesions on the dorsal fin, flank or peduncle. Permanent marks by definition are likely to last many years, enabling long-term identification of these dolphins. In contrast, temporary markings, such as superficial tooth rakes may fade within a relatively

short period of time and inter-annual re-sighting probabilities of these animals are likely to be reduced.

Figure 4.2 Examples of bottlenose dolphin fins showing the three grades of mark severity used in photograph analysis. Each dolphin was graded from one to three as follows: (A) grade M1 marks, consisting of significant fin damage or deep scarring that were considered permanent; (B) grade M2 marking that consist of deep tooth rakes and lesions, with only minor cuts present; (C) fin with grade M3 marks, having only superficial rakes and lesions. Grade M1 (and to some extent, M2) are considered to last many years, enabling long-term identification of these dolphins. In contrast, 'superficial' markings (grade M3), such as tooth rakes may fade and heal within a relatively short period of time and inter-annual re-sighting probabilities of these animals are likely to be reduced.

Digital photographs of dolphins were processed following methods described by Englund *et al.* (2007). For each encounter, the best quality picture was chosen of each identifiable dolphin and the quality of the photograph was graded from 1 to 4 (1 being the best) with no consideration concerning the degree of marking of the dolphin (Table 4.1). Each photographed individual was then assigned one of three grades of mark-severity (Fig. 4.2), and visually matched against the full catalogue/archive of dolphins photographed during previous encounters.

To minimise bias in photograph selection (tendency to favour photographs of well-marked animals due to their distinctiveness), the photographs were graded on their quality first before assigning marking severity grades and only the photographs of sufficient quality (Q1-3) were selected for the abundance estimation. After this, only the "well-marked" dolphins (M1) identifiable from both the left and the right side were selected in order to avoid errors in identification. Photographs from different encounters were compared within and between the regional study sites (i.e. Connemara, Mullet peninsula and Donegal Bay) separately for 2013 and 2014 to establish whether individuals were seen across the whole study area during each year. In addition, a discovery curve was fitted in order to investigate the rate at which

newly identified dolphins were added to the photo-id catalogue during the study period. Over this length of time the impact of changes in marks will be pronounced and the value of heavily marked animals emphasized.

Table 4.1 Scoring criteria for the quality of identification photographs (from Englund *et al.* 2007).

Grade	Criteria
Q1	Well lit and focused photograph taken perpendicular to the dorsal fin at close range
Q2	More distant and less well lit and/or focused or slightly angled photograph of the dorsal fin
Q3	Poorly lit or to some extent out of focus photograph, or a photograph taken at an acute angle to the fin
Q4	Poorly focused, backlit or angled photograph taken at long distance to the animal

Multi-site abundance estimation

Mark-recapture is a widely applied numerical tool in ecology to estimate the number of identifiable individuals in a population (Otis *et al.* 1978). Models, such as M_{th} (Chao *et al.* 1992), that assume population closure on a single site, are typically used in the mark-recapture abundance estimation of dolphins (*e.g.* Wilson *et al.* 1999; Read *et al.* 2003; Ingram *et al.* 2009). Even though the assumption of closure is often violated due to temporary or permanent movement of animals, it is possible to minimise this bias by decreasing the length of the sampling period. However, when animals are encountered in multiple discrete sites during a field season, these methods are no longer suitable as it is often impossible to sample throughout the whole range of the community during a single season, and this can lead to underestimation of the population size (Durban *et al.* 2005). Conversely, combining abundance estimates calculated separately for each site could lead to double counting of individuals due to movements between sites and thus to inflated abundance estimates due to unquantified dependency between sites.

Due to the wide and discontinuous combined survey area and sometimes opportunistic nature of the data collection (when encounters were a result of responses to sighting reports) combined with the large scale movements of the dolphins, we applied Bayesian inference to a model of hierarchical log-linear likelihood of counts of

identified dolphins across multiple discrete sites, and derived a combined abundance estimate of dolphins using the entire survey area (Connemara – Mullet peninsula – Donegal Bay) separately for the years 2013 and 2014. This method developed by Durban *et al.* (2005) is well-suited for sparse data sets (with low number of individual re-sightings) often associated with cetacean mark-recapture sampling and for situations when it is unfeasible to do systematic surveys covering the entire area where the animals are ranging (Durban *et al.* 2005). The model also takes into account the geographical dependencies between the different sites due to for example distance, thus enabling the estimation of the extent of movement between sampling locations. This approach was previously used by Cheney *et al.* (2013) in the abundance estimation of bottlenose dolphins around the entire Scottish coast. An advantage of using Bayesian inference instead of traditional frequentist statistics, on the other hand, is that prior knowledge of the parameter distribution can be incorporated into the model thus producing a joint posterior distribution for the parameters in question. An example of this would be setting a realistic maximum value to the prior for the unseen well-marked animals in an area. This informative prior is then incorporated into the model to facilitate the convergence of Markov Chain Monte Carlo (MCMC) chains.

A contingency table of the sightings histories of identified marked bottlenose dolphins was created based on their presence or absence in each of the study blocks during each season (see Table 4.2) and implemented in the model. The resighting of individuals in multiple survey sites thus represents "capture-recapture events" defined by space instead of time in the context of traditional capture-recapture abundance estimation (Durban *et al.* 2005). The missing value (NA) on the last row of Table 4.2 represents the number of individuals that were not seen in any of the study blocks (missed well-marked dolphins) during the season, and the purpose of the model is to predict a value for the missing cell and thus estimate the overall abundance of well-marked animals across all of the study sites. Specifically, the cell counts (Table 4.2) are treated as independent Poisson random variables with a mean μ_i, so the logarithm of the Poisson mean can be modelled as additive regression function of study area effects:

$$\log(\mu_i) = \beta_0 x_{i0} + \beta_1 x_{i1} + \beta_2 x_{i2} + \beta_3 x_{i3} + \beta_4 x_{i1} x_{i2} + \beta_5 x_{i1} x_{i3} + \beta_6 x_{i2} x_{i3}$$

where β is the vector of unknown parameters to be estimated and the x_i's are indicator variables for the study block classifying factors in the design of the contingency table (Durban *et al.* 2005). For example, $x_{i0} = 1$ for all *i*, and β_0 thus equals to an overall

mean of the counts on the logarithmic scale. The indicators x_{i1}, x_{i2}, x_{i3} then take values of either 1 or -1 depending on the attribute 1 = seen, or -1 = not seen, for study blocks 1, 2, and 3 (or Connemara, Mullet Peninsula and Donegal Bay), respectively (see Table 4.2). The parameters β_1, β_2, β_3 thus denote the main effects of each of the study areas on the overall mean β_0, describing the difference between the average of the μ_i's for cells relating to each study block, and the average of all μ_i's. The terms containing products of any two of these indicators describe two-way interaction effects, with β_4, β_5, and β_6 reflecting the strength and direction of movements of animals from pairs of study sites 1:2, 1:3, and 2:3 respectively. Different models can be produced by omission of one or more of these interaction effects, and so a model averaged estimate for the total number of well-marked individuals, weighted by the relative probability of the candidate models, was produced. This model averaging and prediction were performed using MCMC sampling in WINBUGS software (Lunn *et al.* 2000) with 100,000 burn-in followed by 100,000 iterations. Three chains were run in order to confirm consistency between runs and inspected visually for convergence. The model also incorporates the proportion of well-marked individuals as a binomial sample of the total number of all animals seen (regardless of marking severity); therefore it predicts the number of all individuals in the study area (see Cheney *et al.* 2013).

Table 4.2 Contingency table showing the count of well-marked (M1) bottlenose dolphins present (1) or absent (-1) in each of the study sites Connemara, Mullet peninsula and Donegal Bay in 2013 and 2014.

Year 2013

Count	Connemara	Mullet peninsula	Donegal Bay
3	1	1	1
6	1	1	-1
11	-1	1	-1
11	1	-1	-1
2	-1	1	1
4	1	-1	1
22	-1	-1	1
NA	-1	-1	-1

Year 2014

Count	Connemara	Mullet peninsula	Donegal Bay
8	1	1	1
2	1	1	-1
13	-1	1	-1
6	1	-1	-1
28	-1	1	1
11	1	-1	1
23	-1	-1	1
NA	-1	-1	-1

As a further attempt to describe the ranging behavior of bottlenose dolphins sighted on the west coast of Ireland, the range of sighting latitudes were plotted of the 75 most sighted (≥5 times) well-marked dolphins encountered since the photo-identification catalogue for these animals was started in 2001.

Program TRENDS (Gerrodette 1993) was used to conduct a power analysis in order to assess the amount of sampling effort (in this case, number of years) required to detect a yearly decline of 10% in population size using the coefficients of variation (CV) obtained for the derived abundance estimates. In addition, the effect of different amount of sampling effort on the minimum detectable overall decline in population size was examined with CVs varying from 0.01 to 0.30. Specifically, scenarios were tested when the population was sampled once, every three years, every two years or every year, during a period of six years (which is the reporting period set in the Habitats

Directive). In all the power analyses, the probability of Type I and II errors was set to 0.05, and a one-tailed test was used, as the purpose was to detect a decrease and not a general change in abundance. The CV was chosen as proportional to the square root of abundance (as recommended by Gerrodette 1987 for mark-recapture sampling studies) and an exponential population model used as per Daura-Jorge *et al.* (2013).

4.3. Results

Survey effort and encountered bottlenose dolphin schools

Survey effort varied between years and sites (Table 4.3) with total number of 174 survey hours in 2013 and 146 survey hours in 2014. The number of encounters also varied between sites and years, with eight bottlenose dolphin schools encountered in Connemara, three in the waters around Mullet peninsula and two in Donegal Bay in 2013. In contrast, six dolphin schools were encountered in Connemara, seven around the Mullet peninsula, eight in Killala Bay/south Donegal in 2014 (Fig. 4.3). Bottlenose dolphin school size varied between encounters, locations and years, with larger median school sizes observed in 2014 (Table 4.4). The largest schools were encountered in Donegal Bay with median sizes of 39 and 36 in 2013 and 2014, respectively.

Abundance and movements

Abundance estimates were calculated for each year separately. A total of 59 well-marked bottlenose dolphins were selected for the analysis as identified from the high-quality photographs taken in May–August 2013 (Table 4.1). Fifteen of these individuals (25%) were recorded in more than one of the study areas, with similar numerical overlap between Connemara and Mullet peninsula (six dolphins), Mullet peninsula and Donegal Bay (two dolphins) and Connemara and Donegal Bay (four dolphins). Three out of the 59 well-marked dolphins (5%) were seen in all of the study blocks (Table 4.2). Posterior model probabilities with site interaction terms are presented in Table 4.5., and the model averaged median estimate for the abundance for the whole study area in 2013 was 145 (CV = 0.30, 95% HPDI = 111–239). This estimate includes all well-marked, less marked and unmarked individuals (excluding calves) thus representing the total abundance of dolphins.

Table 4.3 Yearly survey effort in bottlenose dolphin surveys 2013-2014.

	Survey effort (h)	
	2013	*2014*
Connemara	118	68
Mullet peninsula	29	28
Donegal Bay	27	50
Total	***174***	***146***

From the photo-identifications from May–September 2014, a total of 91 well-marked dolphins were included in the analysis (Table 4.2). Eight dolphins (9%) were encountered in all of the study areas. The highest overlap in site use was between Mullet peninsula and Donegal Bay with 28 dolphins (31%) sighted in both these areas. Donegal also had the highest number (23 individuals) of animals seen in only one of the three study sites. The Bayesian multi-site abundance median estimate of the total number of dolphins for the whole study area for the summer 2014 was 189 (CV = 0.11, 95% HPDI = 162–232).

Table 4.4 Median school sizes (with minimum and maximum) of bottlenose dolphins encountered during abundance surveys in 2013-2014.

	School size	
	2013	*2014*
Connemara	8.5 (4 - 25)	19 (11 - 29)
Mullet peninsula	17 (10 - 31)	29 (29)
Donegal Bay	39 (30 - 48)	36 (9 - 95)
All areas	***12 (4 - 48)***	***23.5 (9 - 95)***

The number of well-marked (M1) individuals photographed on the west coast of Ireland between 2013 and 2014 is presented as a discovery curve in Fig. 4.3. The gradual levelling off of the rate of discovery of previously uncatalogued animals could indicate that most individuals occurring within the area were photographed and archived, with fewer new individuals being added towards the end of the sampling period.

Table 4.5 Posterior model probabilities, *P*, of eight log-linear candidate models corresponding to the inclusion of different sets of interaction terms ($\beta_i x_{ii}$) between study sites Connemara, Mullet Peninsula and Donegal Bay. The averaged model was then used to derive abundance estimates for bottlenose dolphins for 2013 and 2014 across all the models.

		P (model)	
Model	Model terms	2013	2014
No interaction – only main effects	$\beta_1 x_{i1}, \beta_2 x_{i2}, \beta_3 x_{i3}$	0.07	0.37
Connemara:Mullet penisula	$\beta_4 x_{i1} x_{i2}$	0.52	0.16
Connemara:Donegal	$\beta_5 x_{i1} x_{i3}$	0.02	0.12
Mullet penisula:Donegal	$\beta_6 x_{i2} x_{i3}$	0.16	0.07
Connemara:Mullet penisula, Connemara:Donegal	$\beta_4 x_{i1} x_{i2}, \beta_5 x_{i1} x_{i3}$	0.03	0.14
Connemara:Mullet penisula, Mullet penisula:Donegal	$\beta_4 x_{i1} x_{i2}, \beta_6 x_{i2} x_{i3}$	0.13	0.06
Connemara:Donegal, Mullet penisula:Donegal	$\beta_5 x_{i1} x_{i3}, \beta_6 x_{i2} x_{i3}$	0.01	0.05
Connemara:Mullet penisula, Connemara:Donegal, Mullet penisula:Donegal	$\beta_4 x_{i1} x_{i2}, \beta_5 x_{i1} x_{i3}, \beta_6 x_{i2} x_{i3}$	0.05	0.03
Averaged		1.00	1.00

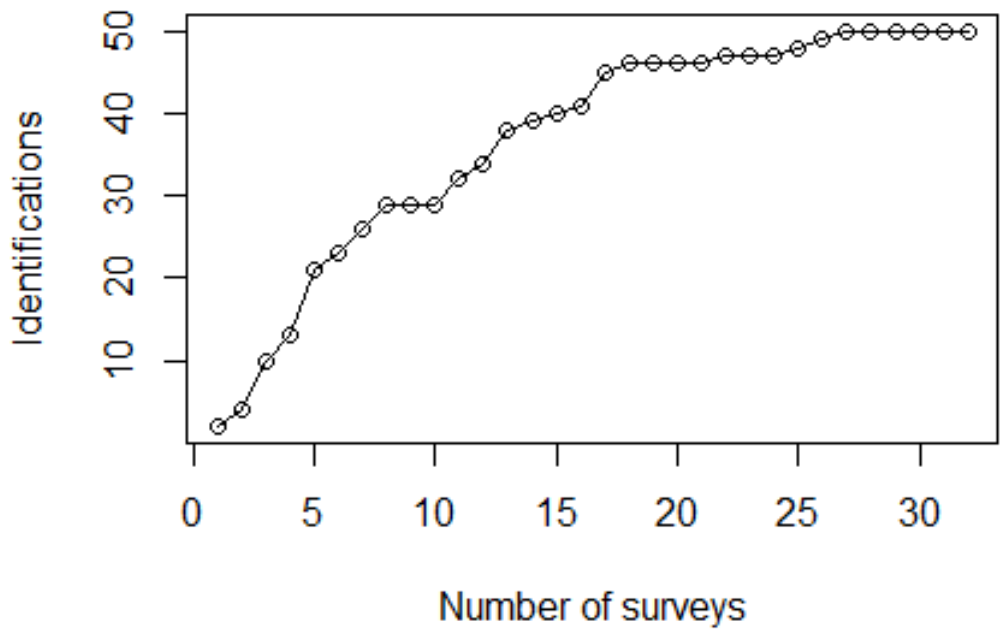

Figure 4.3 Discovery curve for bottlenose dolphin identifications made during photo-identification surveys in 2013–2014. Only well-marked (M1) dolphins identifiable from both sides are included in the data.

The range of sighting latitudes of the most sighted well-marked dolphins identifiable from both sides is presented in Fig. 4.4; it appears that while most of these animals were sighted from Donegal Bay to Connemara with the distance between the areas of nearly 250km, there were some animals that had even wider distribution having been sighted from Co. Cork to Donegal Bay between 2001 and 2014 with over 500km between these sites. In contrast, there were also a few individuals that were only seen in the Connemara study block.

The results from power analysis showed that with a coefficient of variation (CV) of 0.11, the uncertainty associated with the 2014 abundance estimate, it would take six years of annual survey effort to detect a trend of 10% annual decline in abundance with 95% probability; this equates to 47% overall population decline. Moreover, with the larger uncertainty around the 2013 estimate (CV = 0.30), it would require 9 years of annual surveys to detect the same annual trend; this would total to 61% overall decline. The effects of different sampling schemes and CVs on the minimum detectable change in population size are presented in Fig. 4.5. If abundance was sampled twice, as in this study but assuming uniform sampling periods instead of sampling on consecutive years, the minimum detectable overall change in a population during a six year monitoring period would be 29% with a CV of 0.11 (Fig. 3). With the CV of 0.30, however, the population would have to decline to less than 50% of its original level before it could be detected with 95% certainty (Fig. 4.5).

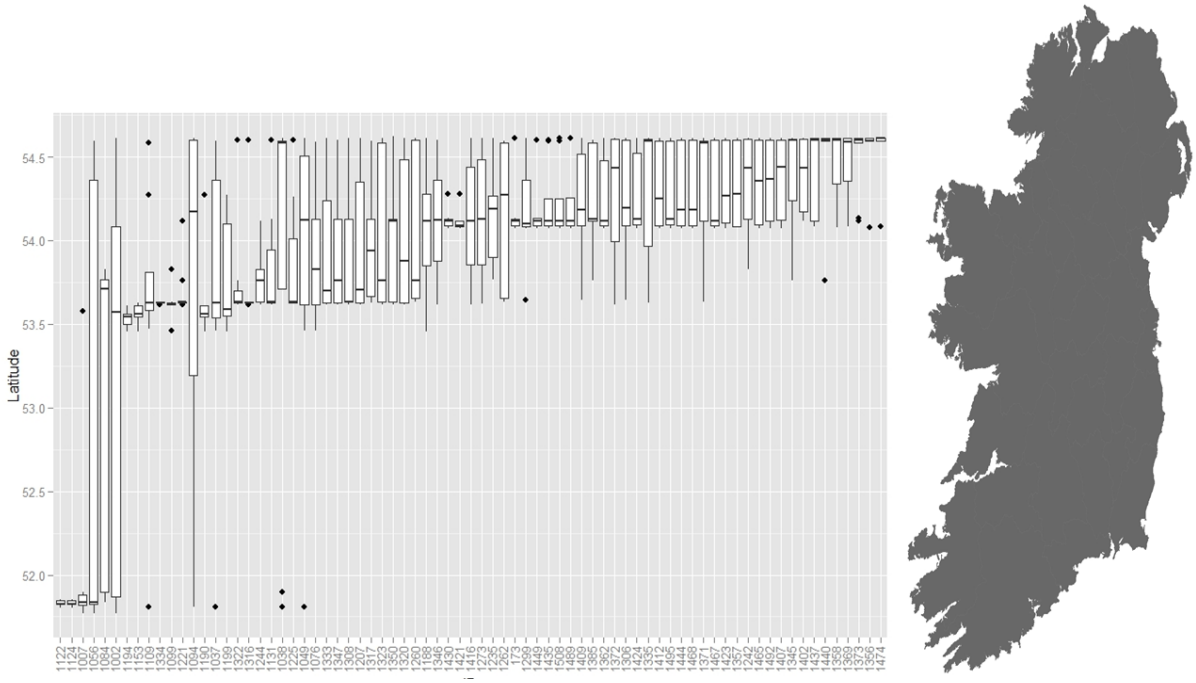

Figure 4.4 The geographic range of the 75 most sighted individually identified bottlenose dolphins. The outline of Ireland is given only for reference and has been scaled to correspond the sighting latitudes. Data for the figure were collected 2001-2014 with only individuals sighted at least five times included. The center line and the bottom and top of the box represent the 50th, 25th and 75th percentiles, respectively, and the whiskers the 5th and 95th percentile. The dots represent "outliers" in the data. The data have been ordered with increasing median latitude.

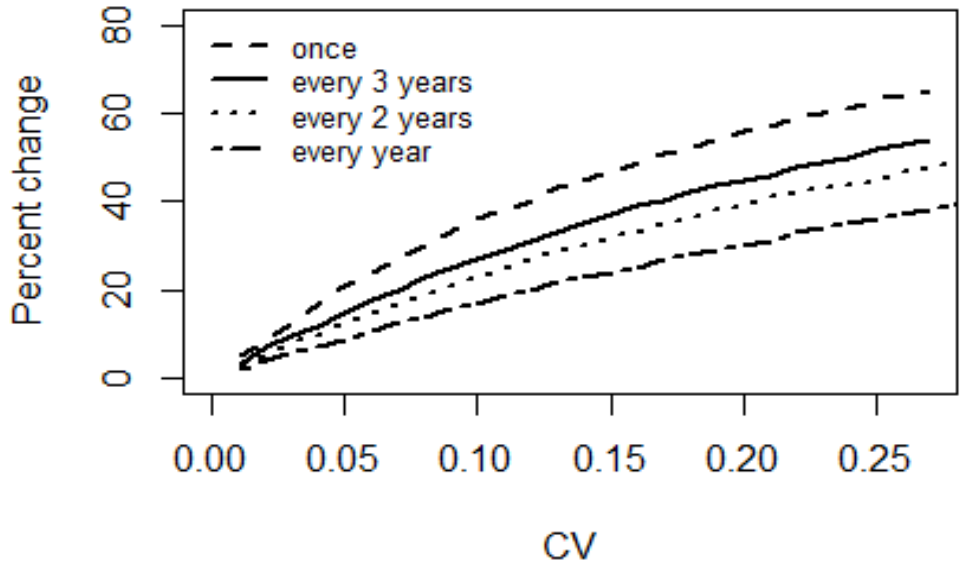

Figure 4.5 The effect of coefficient of variation (CV) to the minimum detectable change in the abundance of a theoretical population with different levels of sampling effort spread uniformly during a 6-year period.

4.4. Discussion

During 2013–2014 survey seasons 83 new dolphin identifications from photographs obtained from both sides of the animal were added to the catalogue. In addition, 38 animals were matched to animals identified from encounters made in previous years with ten identifications dating back as far as 2001 (Ingram *et al.* 2001). Such long term re-identifications indicate that a degree of site fidelity is evident in animals using coastal waters in the west and north-west of Ireland, and it appears that together the combined area between Connemara, the Mullet peninsula and Donegal Bay form an important part of the home-range for a large number of bottlenose dolphins. Some of the dolphins used the entire study area during the two years of survey effort, with almost half of all well-marked identified animals encountered in more than one of the three survey blocks during 2013–2014. This wide scale habitat use pattern produces patchy temporal site occupancy as individuals and schools range freely over considerable distances around the Irish coast and possibly further afield.

Abundance of bottlenose dolphins on the west coast of Ireland

Of the two years of the study, summer estimates of abundance for 2014 were larger and more precise than for 2013 with an overall median multi-site estimate of 189 (CV = 0.11, 95% HPDI = 162–232) compared to 145 (CV = 0.30, 95% HPDI = 111–239), respectively. This difference in the yearly estimates, albeit not significant, may be caused by a combination of environmental, sampling and/or behavioural factors. The total number of surveys, encounters and identifications were higher during 2014 leading to a more comprehensive dataset, and the weather was notably better during that summer than in the previous year. However, the difference may also be attributable to unknown differences in the ranging behaviour of many of the dolphins in the areas surveyed during the two years due to heterogeneous and unpredictable changes in seasonal site use or possibly also due to variation in the composition of the population occupying Irish coastal waters from one summer to the next. Overall, the multi-site abundance estimates, especially the one for the summer of 2014, are comparable with a previous abundance estimate of 171 (95% CI = 100–294) for Connemara for 2009 (Ingram *et al.* 2009) and the cumulative number of animals (N = 179) identified in Mayo in 2008-2009 (Oudejans *et al.* 2010). However, even though the 2014 estimate can be considered reliable, it is likely that the actual number of

animals using the coastal waters of western Ireland falls to the higher side of the confidence interval based on a cumulative number of identifications collected by Ingram *et al.* (2001, 2003, 2009) and M. Oudejans who keeps a separate catalogue of the dolphins occurring in the waters around the Mullet peninsula (M. Oudejans, personal communication).

Abundance of bottlenose dolphins in other coastal areas of the Northeast Atlantic region

With the abundance estimate for dolphins using the Shannon Estuary SAC ranging from 114 to 140 (Berrow *et al.* 1996, 2012; Ingram & Rogan 2002; Ingram & Rogan 2003; Englund *et al.* 2007, 2008), the multi-site abundance estimates for the west coast of Ireland are similar to the ones for the East coast of Scotland obtained using the same multi-site approach (195 with 95% HPDI of 162–253 in 2006, and 227 with 95% HPDI of 175–384 in 2007) (Cheney *et al.* 2013). Another adjacent community of bottlenose dolphins are found in the Sound of Barra, in the Outer Hebrides, Scotland, but this community is significantly smaller consisting of only 6–15 individuals (Grellier & Wilson 2003). This group of dolphins is also thought to have high site fidelity with repeated identifications of the same individuals within and between years. Moreover, Cheney *et al.* (2013) calculated the combined abundance for the entire Scottish Western Coast, including the Sound of Barra, as 45 individuals (95% HPDI of 31–71 in 2006, and 33–66 in 2007). Yet another neighbouring semi-resident group of bottlenose dolphins is found in Cardigan Bay, Wales, with mean summer abundance estimates of dolphins using the SAC varying between 70 and 214 in 2003-2007 (Ugarte & Evans 2006; Pesante *et al.* 2008; Veneruso & Evans 2012; Feingold & Evans 2013). Similar to the 'mobile' population on the West Coast of Ireland, these dolphins are known to occupy a wider range of habitats whilst having a seasonal occupancy in Cardigan Bay during the summer months and majority of the animals moving northwards to the Irish Sea during winter (Baines *et al.* 2002; Pesante *et al*, 2008). Interestingly, based on evidence from a recent study by Louis *et al.* (2014), all of the above mentioned bottlenose dolphins (including the ones on the west coast of Ireland) may belong to a wider 'Coastal North' population thus retaining significant gene flow between the different communities. However, the 'Coastal North' population differs genetically from the much larger but also somewhat nearby 'Coastal South' population occupying the Normano-Breton Gulf of the English Channel whose

abundance was estimated as 420 dolphins (95% CI = 331–521) in 2010 (Louis *et al.* 2015), making this the largest community of coastal bottlenose dolphins in central-northern Europe.

Multi-site model

The multi-site estimates derived in this study are likely to better reflect the true abundance of the coastal bottlenose dolphins on the west coast of Ireland than previous local site-based estimates due to the wider-scale sampling over a larger coastal area. More widespread sampling effort increases the probability of encountering more of these mobile animals and accounts for the pseudoreplication of individuals using multiple surveyed sites if abundance estimates were to be derived separately for each study site. Furthermore, individual heterogeneity in capture probabilities is estimated by geographical dependencies between study sites, included in the model matrix and thus incorporated in the abundance estimate (Durban *et al.* 2005). However, like the M_{th} model by Chao *et al.* (1992), a common method used in cetacean abundance studies (*e.g.* Bearzi *et al.* 2008; Vermeulen & Cammareri 2009; Gnone *et al.* 2011; Brown *et al.* 2014), the Bayesian multi-site approach assumes population closure with no births, deaths, immigration or emigration occurring in the area during the study period (Durban *et al.* 2005). It is likely that although this assumption may be susceptible to violation due to the large scale of the animals' ranges, the inclusion of multiple sites over a broad geographical area should improve this model's performance compared to closed population Maximum Likelihood derived abundance estimates such as the M_{th} model. Furthermore, the short duration of the annual survey season (May–August in 2013 and May–September in 2014) likely reduces the rates of immigration, emigration and deaths of individuals sampled thus increasing the likelihood of effective closure of the sampled population.

Range and movements of individuals

Some of the bottlenose dolphins that were encountered during surveys in 2013–2014 had previously been recorded as far south as Co. Cork and appear to range widely around the west coast of Ireland and possibly beyond (Fig. 4.4). A case in point, a dolphin encountered in Donegal Bay in the summer of 2014 has previously been photographed in the Moray Firth in 2001 and around the Hebrides in 2004 (Robinson *et al.* 2012) thus providing further evidence of the long distance movements and

transient behaviour of at least some of these animals. The dolphins used the entire study area covered during the two years of survey effort, with nearly half (43%) of all well-marked identified animals sighted in more than one of the three survey blocks during 2013–2014. Similarly, Cheney *et al.* (2013) found a large percentage of dolphins (57%) using more than one study site, however, distances between the sites on the east coast of Scotland were shorter compared to the present study with over 300km between Connemara and Donegal Bay.

Management implications

Bottlenose dolphin populations using coastal environments are at particular risk of exposure to a number of anthropogenic threats which may directly impact individuals, for example through disturbance or damage to health and to the overall functioning of the coastal ecosystems upon which they depend. The sensitivity of bottlenose dolphins to some of these threats is exacerbated by their position as an apex predator (Jepson *et al.* 2016) and also by their low reproductive rates (Connor *et al.* 2000; Quick *et al.* 2014). The main threats in coastal environments include pollutants such as xenobiotic chemicals (especially PCBs and DDTs) (Jepson *et al.* 2016), reduced prey availability, habitat degradation, disturbance from vessel traffic (Lusseau *et al.* 2009; Williams *et al.* 2009; Pirotta *et al* 2015), entanglement and incidental bycatch, direct hunting, marine construction and anthropogenic noise (Hammond *et al.* 2012; Williams *et al.* 2014; Pirotta *et al.* 2015). The determination of impacts from anthropogenic habitat degradation on coastal populations requires detailed understanding of the population structure and size of the communities/populations affected, and the ranging behaviour and site fidelity of individuals within these populations. The fact that coastal bottlenose dolphin populations often display fine-scale genetic structuring (Natoli *et al.* 2005; Parsons *et al.* 2002, 2006; Baird *et al.* 2009; Rosel *et al.* 2009; Fernández *et al.* 2011; Martien *et al.* 2011; Mirimin *et al.* 2011; Caballero *et al.* 2012; Gaspari *et al.* 2013, 2015; Louis *et al.* 2014), even in adjacent coastal areas (see Chapter 3; Mirimin *et al.* 2011) where there are no obvious physical barriers preventing gene flow, presents an added challenge to effective conservation and management since delineation of MUs is required and the amount of gene flow between them needs to be quantified before monitoring can commence.

Efficient and regular monitoring of abundance is vital to the management of coastal SACs that have been designated for bottlenose dolphins. Whereas other SACs

designated for the protection of this species around Ireland and the UK appear to describe a considerable degree of site fidelity in a single confined bay (*e.g.* Shannon estuary, Sound of Barra, Cardigan Bay, Moray Firth) this mobile west/north-west coast population does not fit this pattern and thus represents challenges to designing an effective and robust monitoring strategy. Such a strategy must provide accurate data that will contribute to knowledge of the status of the conservation feature and also enable the competent authority to detect, in a timely manner, changes in abundance, population viability and survival rates in order to assess conservation status. The Bayesian multi-site approach used in this study appears to provide a precise and comprehensive estimate of the abundance of dolphins in this wider habitat area and is useful in informing the management of the SAC. With a mobile population with extensive movements such as this one, monitoring the abundance on the dedicated SAC alone would likely lead to underestimation of the true numbers of this population.

In order to be able to detect an overall decline of 25% in abundance over a six-year reporting period, a guideline set in the Article 17 of the Habitats Directive for a population to remain at a "favourable level", the CV around the abundance estimate would have to be as low as 0.08. This would be difficult, if not impossible, to achieve for a mobile population such as the one inhabiting the west/northwest coast of Ireland. An alternative strategy, where the 25% decline could be detected, would be to sample the abundance every two years. However, this would only be achievable if the CV did not exceed 0.11 between sampling occasions. In addition, it was determined that six consecutive years of annual monitoring would be required to detect a 10% annual decline in population, even with a low CV of 0.11. Thus it seems unrealistic to achieve an uncertainty small enough (i.e., CV of 0.02) that an annual decline of 1% (another guideline in Article 17) could be detected. Therefore, regular monitoring and wide-scale research effort where the population is sampled annually or at least every two years is recommended in order to be able to detect changes in population dynamics within this mobile coastal population. This will increase the ability to implement the best possible conservation strategies in a timely manner ensuring the long-term viability of this population.

According to Durban *et al.* (2005) the Bayesian multi-site model of abundance takes into account uncertainty from having a sparse data set and also the uncertainty in model selection by weighing the different model probabilities and thus producing a model-

averaged estimate. It also accounts for much of the individual variation in capture probabilities caused by varying extent of movement of individuals between surveyed sites. However, further heterogeneity unrelated to movement patterns, such as differential reaction of individual dolphins towards the research boat, may still exist that is not captured by the model (Durban *et al.* 2005) so caution should be taken when interpreting the estimates. Nevertheless, the multi-site approach seems to produce abundance estimates with less uncertainty around the point estimate. In addition, multiple discrete locations can be sampled simultaneously, and photo-ID surveys can be done opportunistically with the help from a sightings network prompting the researchers to the location of the dolphins. Monitoring and deriving abundance estimates for a single site is liable to miss animals that range widely and are not present during the period of surveys resulting in negatively biased estimates due to heterogeneity in movements of animals rather than actual changes in population size. In order to gain a more complete picture of the scale of movements and occupancy of this population the work reported should be extended around larger sections of the Irish coast. Such work would ensure that other sites of importance to these animals are identified along with any potentially harmful interactions with human activities.

Although the full extent of the ranges of individuals in this population are not yet known, previous research has shown that at least some of these animals travel distances at the scale of hundreds of kilometres (Ingram *et al.* 2001, 2003; O'Brien *et al.* 2009; Robinson *et al.* 2012; Cheney *et al.* 2013). It seems that these animals may form part of the 'Coastal North' meta-population defined by Louis *et al.* (2014), and trans-national movements of many more individual dolphins than has been reported up to now are likely. High levels of mobility, in turn, can result in substantial gene flow and the homogenization of genetic diversity across a geographic range (*e.g.* Winkelmann *et al.* 2013), and it may be that the transient bottlenose dolphins ranging from the Irish west coast to Moray Firth should be considered as a single management unit and managed in co-operation with Ireland and the UK. As mentioned previously, a number of individuals from the west coast of Ireland have been matched on an *ad-hoc* basis to other existing catalogues but there is a need for a collaborative effort to consistently and regularly compare photo-id catalogues from separate regions/countries (*e.g.* Wales, Scotland, France, Cornwall) in order to better elucidate ranging patterns, demographic dispersal and the abundance of this putative meta-population. In

addition, genetic dispersal within the meta-population needs to be quantified through increased sampling effort over a larger area extending beyond country boundaries and using a common set of genetic markers that are comparable between laboratories. Thus delineating populations remains essential in the management of these dolphins. This is achievable via wider genetic sampling along the entire range of bottlenose dolphins occurring in the coastal areas of Ireland, UK and northern France and with use of a common set of molecular markers. Only after this delineation of MUs can a comprehensive management plans for protecting the populations be drawn.

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Chapter 5: Passive acoustic monitoring of site occupancy by bottlenose dolphins, *Tursiops truncatus*, on the west coast of Ireland

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Abstract

While visual surveys are limited to good weather conditions and visibility, passive acoustic monitoring (PAM) provides a non-invasive method for continuous monitoring of vocalising species. PAM can be used to monitor habitat use, migratory patterns, abundance and foraging behaviour of cetacean and non-cetacean species. The heterogeneous and wide ranging movement patterns of 'mobile' bottlenose dolphins inhabiting the coastal waters of Ireland presents challenges in monitoring. In the present study, temporal and seasonal trends in dolphin occupancy were investigated at two key sites on the west coast of Ireland using dolphin echolocation detection with C-PODs and by applying Generalized Additive Models (GAMs). In addition, the effects of hydrological and environmental parameters that may influence prey distribution were examined in relation to temporal patterns in click detections. Autocorrelation in the data was modelled using Generalized Estimating Equations (GEEs). Tidal current speed and direction were found to be significantly associated with the presence of dolphins with more echolocation click trains logged at intermediate and high current speeds and in northerly and southerly running currents. It may be that the increased detections of dolphins associated with faster current speed reflects the movements of prey species. Significantly more detections were logged by the C-POD deployed at the mouth of Killary Fjord, Co. Galway than by the C-POD in McSwyne's Bay, Co. Donegal. It may be that hydrographic features such as steep and narrow channels in the bottom of the fjord combined with currents resulting from tidal flow may gather and concentrate prey thus facilitating capture in this site. In addition to tidal parameters, an increase in dolphin echolocation click detections in the spring was also recorded in Killary Fjord. This coincides with the peak run of adult salmon, and it is possible that this seasonal increase in prey availability is a driving force for dolphin presence. This study demonstrates the potential of using PAM to reveal information on seasonal and temporal habitat use of dolphins and provides evidence that C-PODs can be used as a long-term monitoring tool in a relatively cost effective way that could potentially be implemented as a part of the monitoring requirements under the EU Habitats and Marine Strategy Framework Directives.

5.1. Introduction

Understanding animal movements, habitat use and their drivers is challenging, especially in wide ranging populations. Environmental and biological processes can influence the relative abundance and distribution of marine organisms on spatial and temporal scales. Marine mammal habitat use can be influenced by a range of drivers including prey availability and foraging strategies (Ford *et al.* 1998; Saulitis *et al.* 2000; Heithaus & Dill 2002; Sveegard *et al.* 2012), anthropogenic disturbance (Johnston *et al.* 2002; Olesiuk *et al.* 2002; Pirotta *et al.* 2014a) and predator avoidance (Heithaus & Dill 2002). Habitat use has also been linked to hydrographic and environmental parameters such as depth, slope, temperature, tidal range and current speed (Wilson *et al.* 1997; Ingram & Rogan 2002; Hastie *et al.* 2003; Hastie *et al.* 2004; Embling *et al.* 2010; Booth *et al.* 2013). Significant drivers of the wide-scale distribution of cetaceans, on the other hand, include at least temperature, primary productivity and ocean fronts (Jaquet & Whitehead 1996; Littaye *et al.* 2004; Kaschner *et al.* 2006; Foote *et al.* 2013; Scales *et al.* 2014, see also Fig. 2.2 in Chapter 2), factors that are susceptible to considerable temporal fluctuations. Understanding temporal and spatial habitat use of marine mammals is often challenging due to the dynamic environment that they live in.

While visual surveys are limited to good weather conditions and visibility, passive acoustic monitoring (PAM) provides a non-invasive method for continuous monitoring of vocalising species that can be elusive or otherwise visually difficult to detect. PAM is commonly recommended for mitigation of impacts on cetaceans during marine construction such as pile driving or activities related to seismic surveys (JNCC 2010; DAHG 2014). PAM can also be used in studies on habitat use, migratory patterns, abundance and even foraging behaviour and depredation of cetacean and non-cetacean species (Norris *et al.* 1999; McDonald & Fox 1999; Carlström 2005; Verfuß *et al.* 2007; Luczkovich *et al.* 2008; VanParijs *et al.* 2009; Rountree *et al.* 2011; Kyhn *et al.* 2012; Marques *et al.* 2013; Pirotta *et al.* 2014b; Thode *et al.* 2015; Benjamins *et al.* 2016).

Types of acoustic monitoring devices used to detect cetaceans vary from extensive static hydrophone arrays such as SOSUS and AUTEC (deployed by the US Navy for submarine surveillance) to single stationary hydrophones and towed hydrophone

arrays (Mellinger *et al.* 2007). These systems usually record sounds on a certain frequency band and can be selected based on the vocalisation range of the target species. They can either collect and store data continuously (like the SOSUS array and various self-contained units such as the archival marine acoustic recording unit (ARU), Ecological Acoustic Recorder (EAR) and Wildlife Computers (SM2M), or they can be set to record for a period of time on a predefined interval. The recordings are either stored in the unit itself which can then be retrieved, or the hydrophone can be linked via a cable to a listening station allowing for real-time analysis of the sound data. Unlike conventional hydrophones, C-PODs (or T-PODs, older versions of C-PODs) are commonly used for detecting and monitoring odontocetes particularly in coastal habitat (Chelonia Ltd, Cornwall, UK). C-PODs are self-contained battery-powered acoustic devices that detect echolocation clicks or bouts produced underwater by toothed whales like dolphins and porpoises mainly for orientation and localization in foraging (see Tyack and Miller 2002). The conservative detection radius of C-PODs has been estimated to be ~300–400m for bottlenose dolphins depending on the behaviour of the animals, but detections up to over 1,500m have been recorded (Nuuttila *et al*. 2013). These autonomous loggers, which are capable of collecting continuous time-seies data for several months between service intervals, do not record sound but instead log the time, duration, inter-click interval, dominant frequency and other features of detected click trains (defined as consisting of at least five consecutive clicks) with up to a 5ms resolution. The click bouts detected and logged by the C-POD are saved onto an SD card, from which the data can be downloaded and analysed. However, even though the filtering algorithms that come with the C-POD software can discriminate between narrow-band high-frequency clicks produced by porpoises and broad-band dolphin echolocation clicks, the identification of individual animals or the effective discrimination of clicks from different dolphin species is not currently possible (Tregenza 2013) due to the high overlap in click train characteristics (Robbins *et al.* 2015). Nevertheless, C-PODs (and T-PODs) provide a reasonably economic way to collect information on the site use of echolocating odontocetes.

The common bottlenose dolphin, *Tursiops truncatus*, has been allocated a special protected status under Annex II and Annex IV of the European Union's Habitat's Directive, which means that the status of the species must be maintained at or restored to a 'favourable condition'. To protect critical areas for the two genetically and socially

distinct coastal populations inhabiting Irish coastal waters (Mirimin *et al.* 2011; Chapters 3 and 4), two Special Areas of Conservation (SACs) for bottlenose dolphins have been designated, one in the Shannon estuary (Lower River Shannon SAC) and one in two areas in Connemara and Mayo (West Connacht Coast SAC) (Fig. 5.1).

The wide geographic ranging patterns of members of the 'mobile' west coast population, estimated to comprise 189 dolphins (median estimate, with a CV of 0.11, see Chapter 3 on abundance), presents significant monitoring challenges. Therefore, in the present study, temporal and seasonal trends in dolphin occupancy were investigated at two key sites on the west coast of Ireland using dolphin echolocation detection with C-PODs. In addition, the effects of hydrological and environmental parameters such as tide height, speed and direction, primary productivity and Sea Surface Temperature (SST), were examined in relation to temporal patterns in click detections and dolphin site occupancy, and the potential of using PAM as a monitoring tool in the management of marine protected areas is discussed.

5.2. Materials and Methods

C-POD deployment and study sites

The two deployment locations in Western Ireland were selected, one at the mouth of Killary Fjord, Co. Galway (part of the West Connacht Coast SAC), and the other in McSwyne's Bay, Co. Donegal (Fig. 5.1.), based on prior knowledge of bottlenose dolphin habitat use (Ingram *et al.* 2001, 2009). Killary Fjord is one of the three glacial fjords situated in Ireland. The fjord is approximately 15km long and 0.75km wide with an average depth of 15m and a maximum depth of 45m. Small scale commercial draft netting fisheries operate in the fjord with an annual catch of 369 salmon reported in 2012 (Anonymous 2012), and the three tributaries feeding into the fjord are important rivers for recreational salmon fishing with the combined harvest of 287 fish in these rivers in 2012 (Anonymous 2012). In addition, Killary Fjord is an important site for shellfish aquaculture, and a salmon farm is situated immediately outside the mouth of the fjord. McSwyne's Bay, on the other hand, is approximately 8km wide with an average depth of 20-35m. Similar to Killary Fjord, McSwyne's Bay is also an important area for aquaculture with salmon and mussel farms in proximity of the C-POD deployment site.

The C-PODs used in this study were deployed very close to shore to minimise the detection of other delphinid species (e.g. Perrin 2002; Bearzi *et al.* 2003), in waters of 6-12m depth (at low tide) and 2-3m of the bottom of the seafloor. The C-POD in McSwyne's Bay, Co. Donegal, was deployed on the 19th of October 2013, and a second C-POD was deployed in Killary Fjord, Co. Galway, on the 15th of September 2014 following a theft of a C-POD previously deployed in early June 2014. The C-PODs were retrieved every 4-6 months for data download and battery replacement (subject to weather conditions) and were re-deployed as soon as possible after the maintenance.

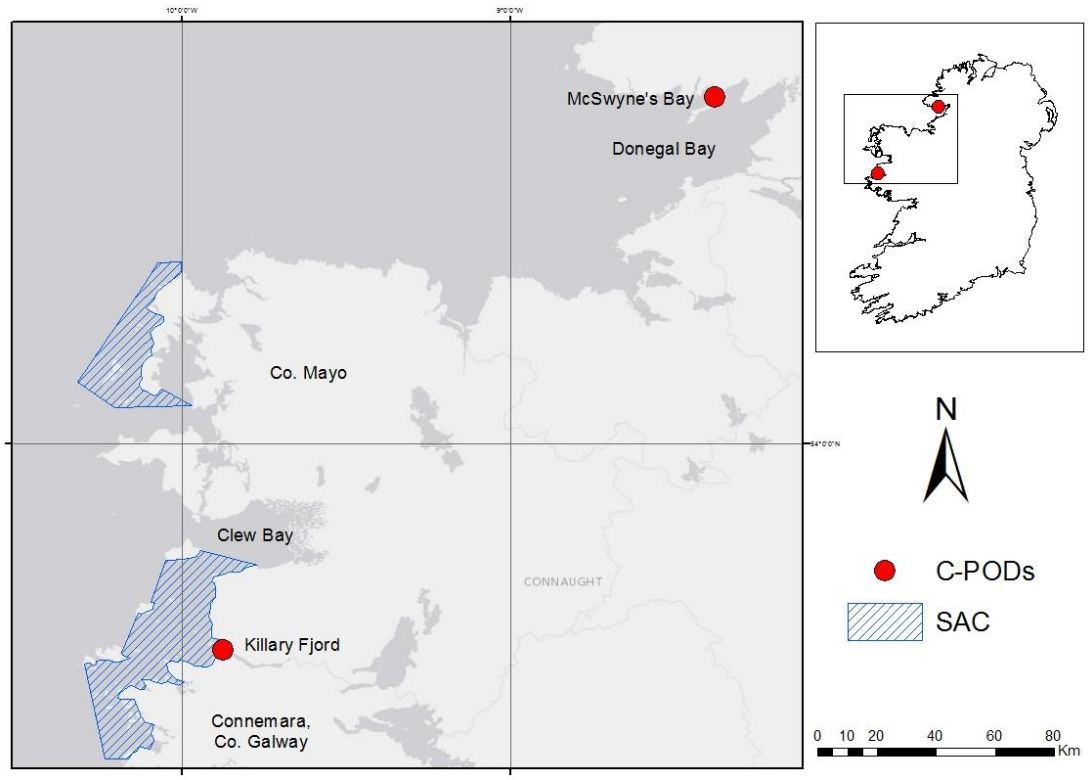


Figure 5.1 Locations of C-PODs deployed to detect bottlenose dolphins using the West Connacht Coast SAC and a previously identified 'hotspot' in Donegal Bay (Ingram *et al.* 2001).

C-POD data were analysed in C-POD.EXE software (Chelonia Ltd.) using the GENENC click classifier and 'other cet' setting which maximises the capture of echolocation click events for dolphins (N. Tregenza, Chelonia Limited, personal communication). The classifier also discriminates broadband dolphin clicks from narrow-band high frequency harbour porpoise (*Phocoena phocoena*) clicks, boat engine noise and background environmental noise such as sounds caused by the moving sediment.

Environmental data

The environmental variables were chosen for this study partly due to their significant effect on dolphin presence found in a previous study (i.e. tidal parameters, Pirotta *et al.* 2014) or due to their potential effects on prey availability driving the distribution of marine predators (Mendes *et al.* 2002). Remotely sensed surface chlorophyll-a data from the MODIS sensor on the NASA's Aqua satellite were downloaded as monthly values averaged over 4km × 4km grid cells within an equivalent area surrounding each of the two deployment sites. These data were downloaded using the GIOVANNI portal on the NASA website (http://giovanni.gsfc.nasa.gov/giovanni/). Real time hourly measurements of Sea Surface Temperature (SST) in Galway Bay were downloaded for the duration of the deployment from the Marine Institute website (http://www.marine.ie/Home/site-area/data-services/real-time-observations/wave-buoys) and used as a large scale proxy for SST at both deployment sites. Time-series data on sunrise and sunset times for the west coast of Ireland (54.451°N, 9.297°W) were retrieved using R (R Core Team 2016) package 'maptools' (Bivand and Lewin-Koh 2016); the functions use algorithms provided by the National Oceanic & Atmospheric Administration (NOAA). Hourly tidal level, tidal current speed and direction were obtained using POLPRED (NERC National Oceanography Centre, Liverpool, UK). However, predictions of tidal information at locations immediately close to the coast are not available in POLPRED; therefore the predictions were made to the closest possible grid cell to the C-POD deployment locations, within 8km from both locations.

Statistical modelling: GEE-GAMs on time-series data

The R (R Core Team 2016) package 'MRSea' (Hayward *et al.* 2013) with Spatially Adaptive Local Smoothing Algorithm (SALSA, Walker *et al.* 2010) was used to fit splines to the continuous covariates in generalized additive models (GAMs; Wood 2006). SALSA is an automated procedure that finds the best way to fit a regression spline for one or two-dimensional covariates and performs knot selection, or otherwise reduces the covariate to a linear term (Walker *et al.* 2010; Hayward *et al.* 2013). Due to different temporal resolution in the environmental data sets, explanatory variables included in the GAMs were divided into three different models: an hourly model, a daily model and a monthly model. The hourly model included a factor covariate 'daylight' with two levels 'light' and 'dark', and continuous tidal covariates 'tidal

level', 'tidal current speed' and 'current direction'; in general, this model included covariates measured on an hourly scale. The response variable was modelled as presence/absence (of dolphin echolocation click trains per hour) with a binomial distribution due to the fact that the model residuals were not over- or under-dispersed. The daily model included the factor variable 'site' (deployment location) and temporal covariates 'year' and 'Julian day', the latter was approximated as a continuous covariate. The response variable 'detection positive minutes per day' (DPM) was modelled with a quasipoisson distribution to account for over-dispersion. In addition, an effort term was included in the model to account for any bias caused by difference in the number of hours per day that the C-PODs were operational due to servicing. Covariates 'temperature (°C)' and 'productivity' (chlorophyll-a, mg/m^3) were included in the monthly model, and the response variable was modelled as detection positive days (DPD) with a quasipoisson distribution again due to overdispersion of the model residuals. Multi-collinearity of the covariates was tested for all of the models by calculating Generalized Variation Inflation Factors, GVIFs (Fox & Weisberg 2002). The continuous variables 'tidal level', 'tidal current speed', 'current direction', 'productivity' and 'temperature' were modelled with cyclic cubic splines, and the circular covariate, 'Julian day', with a b-spline. The different models and covariates have been summarized in Appendix 5.1.

An autocorrelation function (ACF) plot was used to visually check the level of temporal autocorrelation in the, and generalised estimating equations (GEEs; Liang & Zeger 1986) were subsequently applied after fitting the GAMs with the purpose of explicitly modelling the observed autocorrelation within the blocks (see also Dormann *et al.* 2007; Pirotta *et al.* 2011; Pirotta *et al.* 2014; Culloch *et al.* 2016). GEEs can be used to uncover marginal (i.e. population-averaged) effects rather than conditional effects that relate to the estimated effect on an individual (Heagerty & Zeger 2000). With the GEE approach, data points were divided into independent blocks, and a correlation structure for the residuals specified within blocks (Liang & Zeger 1986). One specific benefit of using GEEs in analyzing autocorrelated datasets is that they allow for differences in the level of autocorrelation among blocks (Koper & Manseau 2009). In addition, parameter estimates and empirical standard errors in GEEs are robust even in a situation where the correlation structure might be misspecified, as found by simulation studies (Liang & Zeger 1986; Overall & Tonidandel 2004). Quasi-

likelihood under the model independence criterion (QIC) values (Pan 2001) were used to select the best autocorrelation structure between "working independence", "exchangeable" and "AR1" structures in the binomial hourly model and in the daily model with Poisson distributed data. Repeated Wald's tests were used to assess the significance of the retained covariates (Hardin & Hilbe 2003). Finally, diagnostic residual plots were inspected to assess the fit and predictive power of the best model.

5.3. Results

In total, out of more than 24,000 hours of deployment, the data set collected with both of the C-PODs included 6,600 detection positive minutes where at least one bottlenose dolphin echolocation click train was logged (Table 5.1). The number of detection positive days (DPD) per month in 2013–2015 are shown for both C-PODs in Fig. 5.2. The results show that dolphins were detected in Killary Fjord every month during the deployment with presence on at least 15 days during the months of March–July 2015 (Fig. 5.2), whereas dolphin presence was in general lower in McSwyne's Bay with no detections logged during January–February 2015 (Fig. 5.2).

Table 5.1 C-POD deployment summary showing the number of deployment days per year for each study site, and the number of detection positive days and minutes logged during those days.

	No. of deployment days		Detection positive days		Detection positive minutes	
Year	McSwyne's Bay	Killary Fjord	McSwyne's Bay	Killary Fjord	McSwyne's Bay	Killary Fjord
2013	74	0	14	0	237	0
2014	314	108	81	32	2218	261
2015	260	265	46	152	665	3219
Total	**648**	**373**	**141**	**184**	**3120**	**3480**

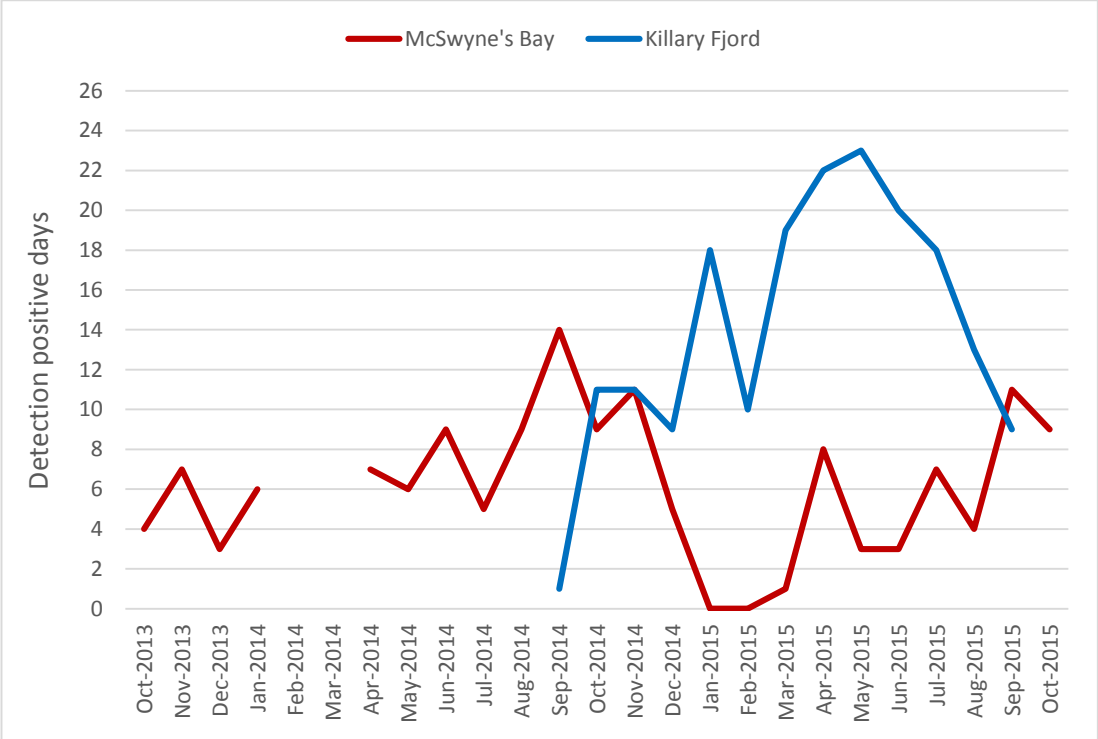


Figure 5.2 Detection positive days (DPD) per month logged by C-PODs during the deployment in 2013-2015. Note that the break in the line means that the C-POD had run out of batteries and was not logging clicks.

Hourly model

The average tidal current speed was 0.04m/s (SD = 0.02) in Killary Fjord, and 0.04m/s (SD = 0.05) in McSwyne's Bay. The mode of tidal current direction was 290° in Killary Fjord and 292° in McSwyne's Bay. Significant positive temporal autocorrelation was found in the hourly data set (Run's test statistic: -66.79, P <0.001), and after inspecting the correlation plot (Fig. 5.3), the data were divided into weekly blocks for which the autocorrelation was modelled using GEEs (Liang & Zeger 1986). The GEE-GAM with "working independence" autocorrelation structure was chosen over the "exchangeable" and "AR1" based on the QIC-values ("independence" QIC: 6993.6, "exchangeable" QIC: 7034.8, "AR1" QIC: 7011.1).

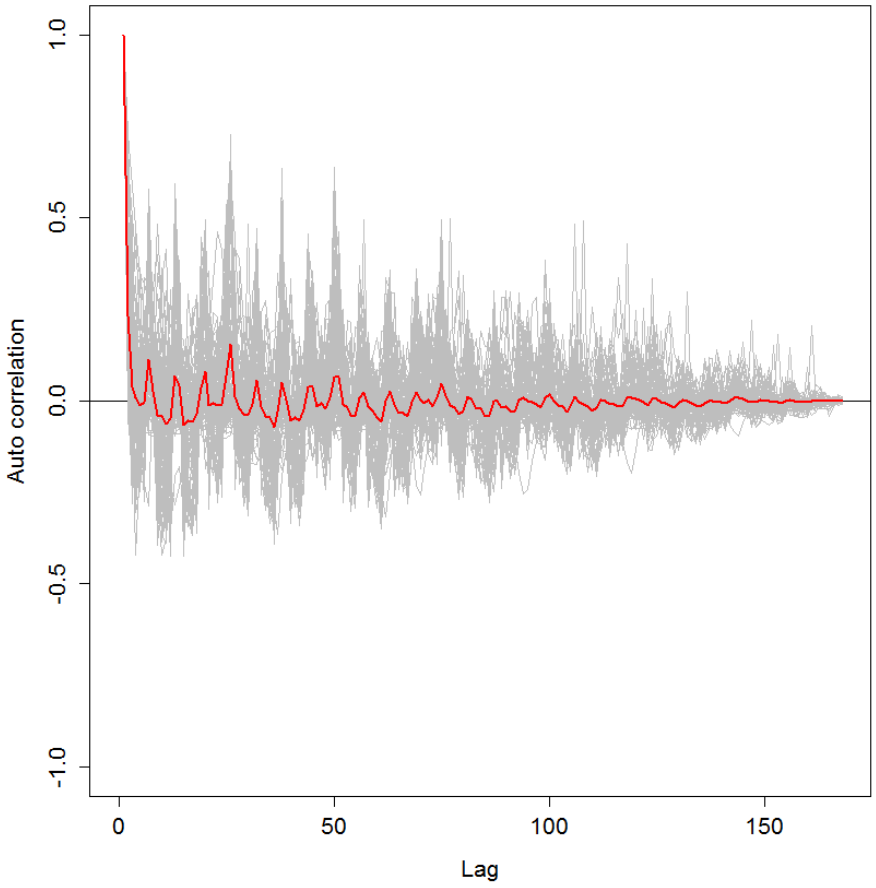
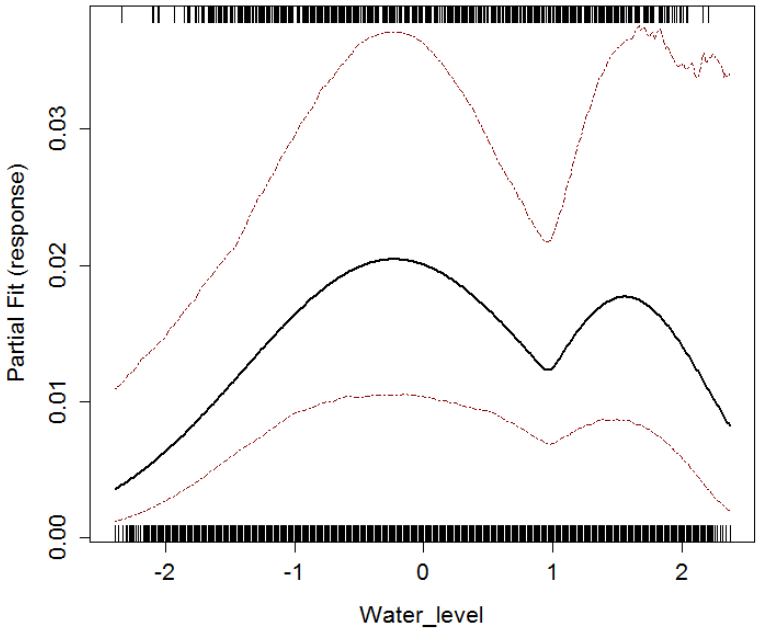


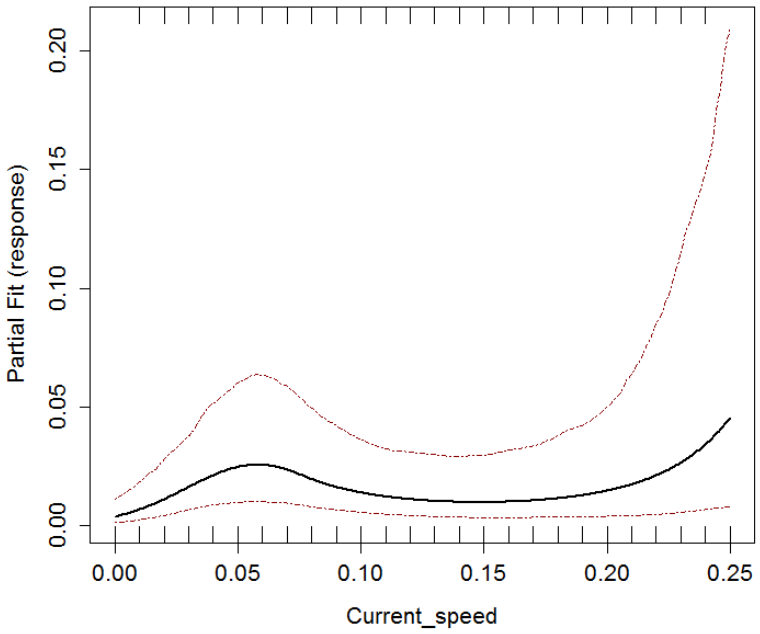
Figure 5.3 Correlation of the hourly GAM residuals for each block (grey lines) and mean autocorrelation (red line) at each lag. Autocorrelation values of >0.05 denote significant autocorrelation. The time lag is given in hours, 150h corresponds to approximately 6 days, after which autocorrelation diminishes.

Significant explanatory variables kept in the best performing GEE-GAM were 'daylight' with an increase in the probability of detections during hours of daylight (P <0.05), 'water level' with the probability of dolphin detections increasing at mid and anf higher tidal levels (P <0.0001), tidal current speed' (P <0.0001) with an observed bimodal effect on the probability of dolphin presence, and 'tidal current direction' (P <0.001) with more detections occurring with northerly and southerly tidal flows (see Fig. 5.4 for the partial residual plots of these variables). The model fit and predictive power were poor with concordance correlation and marginal R^2 values between fitted and observed values of 0.020 and 0.011, respectively, implying that only very a small proportion of variation in dolphin presence was explained by these covariates.

(A)


Partial Fit (response)
0.00
0.01
0.02
0.03
-2
-1
0
1
2
Water_level

(B)


Partial Fit (response)
0.00
0.05
0.10
0.15
0.20
0.00
0.05
0.10
0.15
0.20
0.25
Current_speed

(C)

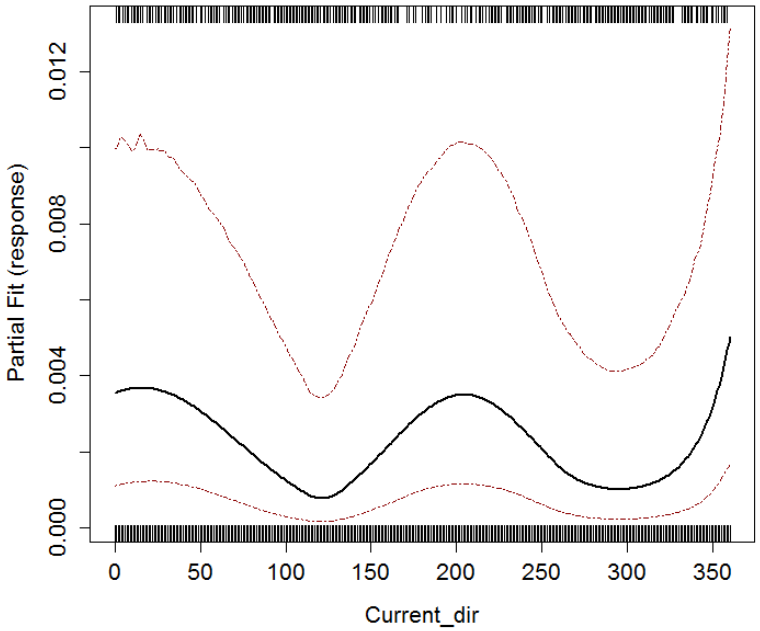


Figure 5.4 Partial residual plots of the best GAM-GEE model for dolphin presence/absence and significant explanatory continuous covariates in the hourly model. (A) Estimated relationship with 'water level'. (B) Estimated relationship with 'tidal current speed'. (C) Estimated relationship with 'tidal current direction'. The dotted lines represent the 95% confidence intervals, and the rug plot on the x-axis shows the actual data values, with probability of presence and absence on the upper and lower part of the plot, respectively.

Daily model

Significant positive autocorrelation (Run's test statistic: -16.69, P <0.001) in the detection positive minutes was modelled by dividing the data into weekly blocks, for which the autocorrelation was modelled using GEEs (Liang & Zeger 1986) with a "working independence" correlation structure, based on the inspection of the GAM residuals (Fig. 5.5) and the QIC values ("independence" QIC: -13128.4, "exchangeable" QIC: -13127.2, "AR1" QIC: -13121.8). Significant covariates kept in the best model were 'site' with significantly more detections logged in Killary Fjord (*P* <0.001) and the interaction between 'site' and 'Julian day' with a bimodal response in McSwyne's Bay (*P* <0.001, 5.6a) with a slight increase in detections at ~100 days (March) and a higher peak at ~270 days (September), and a single peak in the number of detections in Killary Fjord at ~120–150 days, i.e. in April–May (*P*

<0.001, Fig. 5.6b), and. The concordance correlation and marginal R^2 values between fitted and observed values were 0.080 and 0.043, respectively, indicative of poor model fit and predictive power.

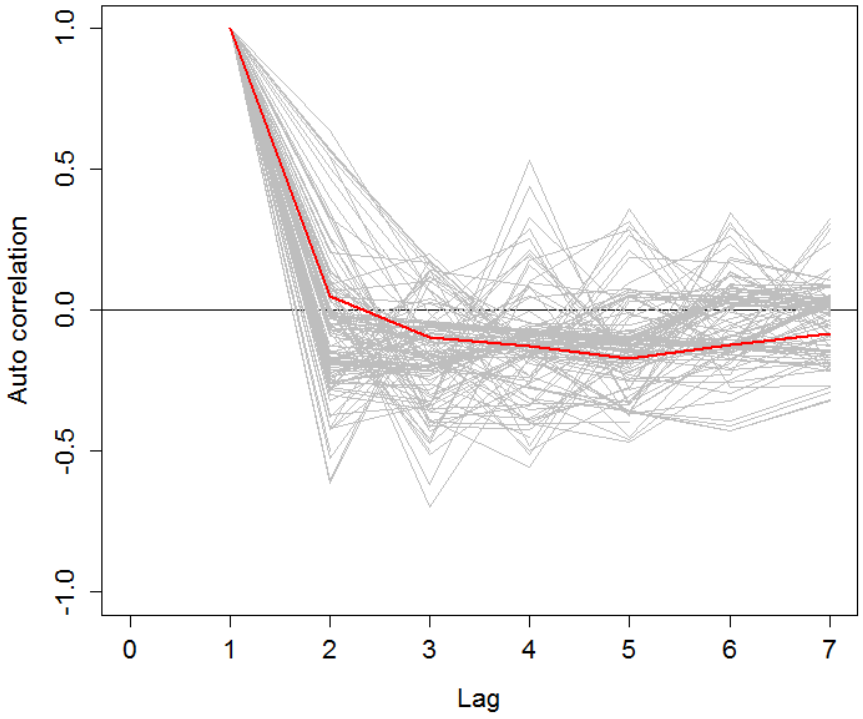
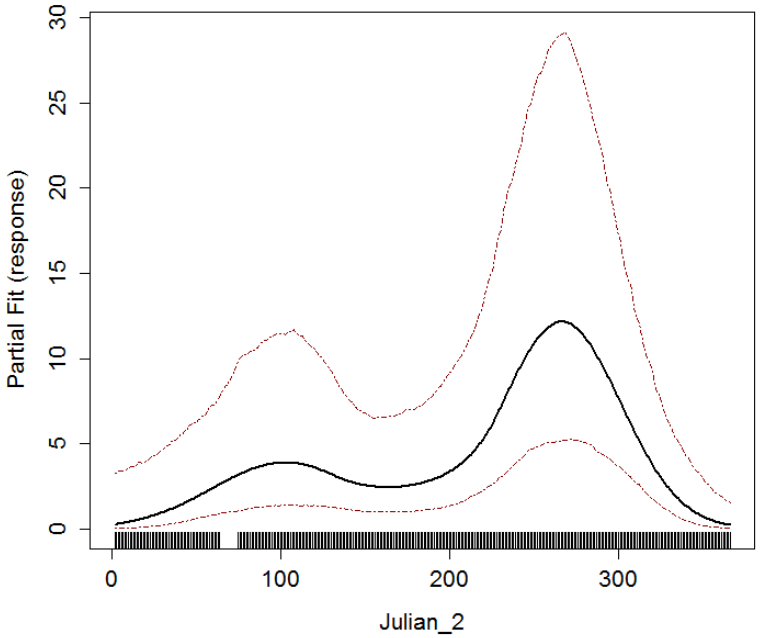


Figure 5.5 Correlation of the daily GAM residuals for each block (grey lines) and mean autocorrelation (red line) at each lag. Autocorrelation values of >0.05 denote significant autocorrelation. The time lag is given in hours, 150h corresponds to approximately 6 days, after which autocorrelation diminishes.

(A)



(B)

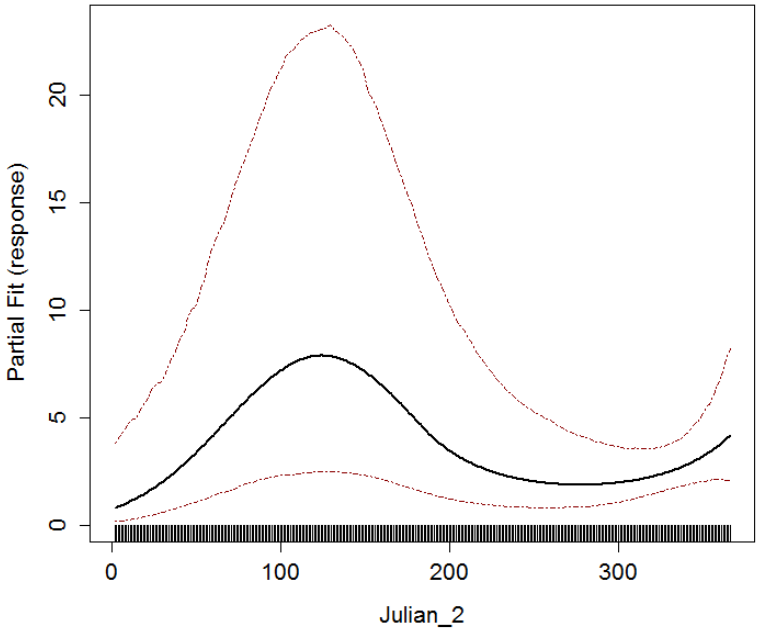


Figure 5.6 Partial residual plots of the best GAM-GEE model for the detection positive minutes and the significant explanatory continuous covariate, 'Julian day', in (A) McSwyne's Bay, Co. Donegal, and (B) Killary Fjord, Co. Galway, in the daily model. The dotted lines represent the 95% confidence intervals, and the rug plot on the x-axis shows the actual data values of counts.

Monthly model

The temperature varied from 6.9°C recorded in February of 2015 to 16.0°C in July of 2014, and productivity measured as chlorophyll-a concentration varied from 0.2 to 17.2 mg/m^3 in the area surrounding Killary Fjord, and from 0.8 to 37.7 mg/m^3 around McSwyne's Bay. No temporal autocorrelation was found in the monthly dataset, therefore, GEEs were not incorporated in the GAM. From the covariates, only 'mean productivity' was kept in the best model but it was non-significant (P = 0.190).

5.4. Discussion

In addition to obtaining information on site occupancy and habitat use of bottlenose dolphins, understanding which environmental factors influence habitat use is important in the initial selection and subsequent monitoring of protected areas. In an attempt to answer these questions, echolocation click detections of bottlenose dolphins at two different locations on the west coast of Ireland were modelled with a number of environmental parameters that have the potential to influence the habitat use of dolphins on different temporal scales.

A significant increase in dolphin detections was found during daylight hours, which can either imply that dolphins were more likely to visit the sites during the day, or that they were more active in producing echolocation clicks during daylight hours. In contrast, a significant increase in occurrence and foraging activity (presence of feeding buzzes, click trains that have short and progressively decreasing inter-click intervals) was recorded during hours of darkness in a study with resident Ocean humpback (*Sousa plumbea*) and Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) in Zanzibar, Tanzania (Temple *et al.* 2016). However, data collected in Sarasota Bay, Florida, have shown that bottlenose dolphins are acoustically active at night as well as during the day and that foraging activities are undertaken during both day and night with echolocation clicks and buzzes occurring throughout the entire 24-hour period (Wells *et al.* 2013). If the (acoustic) activity budgets of bottlenose dolphins on the west coast of Ireland are similar to the ones in Sarasota Bay, the scenario where the dolphins visit these coastal sites more during daylight hours rather than a difference in echolocation production seems more likely, assuming that they are producing echolocation clicks when they are within the effective detection range (300-400m, Nuuttila *et al.* 2013) of

C-PODs. Even though echolocation has been found to occur significantly less frequently when bottlenose dolphins are travelling compared to other activities (Jones & Sayigh 2002; Dos Santos & Almada 2004), echolocation click detections have been found to corresponded well with visual detections, with over 80% of the dolphin schools observed within 500m also detected acoustically (Philpott *et al.* 2007) indicating a high probability of detection due to active echolocation behaviour.

Tidal height and current speed had a significant effect on the number of detection positive minutes with a peak in detections at mid to high water levels and intermediate and fast current speeds. Similarly, in another study, bottlenose dolphins were found to be significantly more abundant during flood tide (i.e. incoming tide), particularly during the stationary stage of the tidal front (Mendes *et al.* 2002). Pirotta *et al.* (2014), however, found no significant effect of current speed on the presence of feeding buzzes. However, occurrence of feeding buzzes was not the focus of this study, and Pirotta *et al.* (2014) did not investigate how well the presence of feeding buzzes corresponded to the presence of echolocation clicks; thus the results in these two studies are not directly comparable. Nevertheless, the significant effect of tidal height and current speed found in this study may be influenced by different tidal flow conditions between the study sites, which in turn may facilitate the presence of different types of prey. It may be that the increased presence of dolphins associated with higher water level and faster current speed found in this study reflects the movements of prey species (Sveegard *et al.* 2012; see review by Benjamins *et al.* 2015). Telemetry studies, although concentrating on larger species such as blue marlin, *Tetrapturus audax*, or blue shark, *Prionese glauca*, have found an effect of currents on fish swimming speed and directionality (Carey & Scharold 1990; Brill *et al.* 1993). Interestingly, Atlantic salmon (*Salmo salar*) smolts have also been shown to take advantage of tidal ebbs during their seaward migration and hold their position during flood tides (Moore *et al.* 1995; Lacroix and Curdy 1996). In addition, adult salmon have also been shown to time their movements with tidal currents in estuaries when returning to breed (Potter 1988). Atlantic salmon is likely to be an important part of bottlenose dolphin diet in Ireland, at least seasonally. Adult fish have been recorded from stomach contents of dolphins stranded around Ireland (Hernandez-Milian *et al.* 2015) and shown to be an important resource for a nearby resident population of bottlenose dolphins inhabiting Moray Firth, Scotland (Janik 2000; Bailey & Thompson

2009). Further, anecdotal evidence exists that salmon fishery catches are higher during spring tides when the current speed reaches its peak. Concordant to the findings by Pirotta *et al.* (2014), tidal current direction was also significant in this study with an increase in echolocation bouts being recorded at currents running from South to North or from North to South (Fig. 5.4b). The tidal flood on the west coast of Ireland has a northerly direction (Anonymous 2004), and this could also influence movements of Atlantic salmon or other prey species.

In addition to tidal parameters, an increase in dolphin echolocation click detections in the spring was also recorded in this study in Killary Fjord. Pirotta *et al.* (2014) found an increase in feeding buzzes coinciding with the summer months in Moray Firth, Scotland. Even though the present study did not distinguish and classify feeding buzzes within the echolocation click bouts, it is likely that they are represented widely in the dataset. In fact, evidence exists that echolocation is used more frequently for feeding than in any other behavioural context (Jones and Sayigh 2002; Nowacek 2005; Gannon *et al.* 2005). Unlike the more northerly parts of Europe, where the timing of the runs of Atlantic salmon is largely dictated by temperature and melting of ice and thus limited to June–September, salmon can be found migrating back up river to spawn almost on any day of the year around the British Isles and Ireland (Sutterby & Greenhalgh 2005, Reed *et al*. 2016). Run timing is one of many life-history traits used to characterise Atlantic salmon, and differences in salmon migration between rivers and even between tributaries has been known for a long time. This variation in run timing is typically associated with time spent feeding at sea with larger multi-sea-winter individuals, which spend two or more years at sea, tend to enter rivers earlier in the year (spring) than smaller fish that have spend only one year at sea (Reed et al. 2016). However, the run peaks predominately occur during the spring (March–April) and summer (June–July) in Irish rivers (Quinn *et al.* 2006). It is thus possible that these peaks in salmon runs, especially the spring peak which is dominated by larger multi-sea-winter individuals (Quinn *et al.* 2006), are the drivers for dolphin presence in certain coastal sites and explain the greater number of detections coinciding with spring months in the Killary Fjord. Increased presence of dolphins linked to salmon run times has also been suggested by other authors (Wilson *et al.* 1997; Mendes *et al.* 2002), and is supported by the fact that River Bundorragha, a tributary of Killary Fjord, contributes to substantial number of spring salmon catches (Reed *et al.* 2016).

Significantly more detections were logged by the C-POD deployed at the mouth of Killary Fjord, Co. Galway than by the C-POD in McSwyne's Bay, Co. Donegal. Killary Fjord and the associated tributaries are some of the most important salmon rivers in Ireland, and this combined with a large number of visual encounters indicates that the fjord is an important foraging area for bottlenose dolphins. Moreover, this is further supported by numerous observations of bottlenose dolphins chasing and capturing Atlantic salmon within the fjord. It may be that hydrographic features such as steep and narrow channels in the bottom of the fjord combined with currents resulting from tidal flow may gather and concentrate prey (salmon and other) thus facilitating capture. The deployment site in McSwyne's Bay, Donegal, on the other hand, is a much wider and more open bay with different bathymetry and hydrographical features compared to Killary Fjord. It is located ~25km north from the closest salmon river, River Eske, and this may be one reason explaining the fewer number of detections logged at this site and the bimodal peak in detections occurring during the winter months.

Temperature was not found to be a significant factor explaining the presence of echolocation click trains logged by the C-PODs, and primary productivity (approximated as chlorophyll-a concentration, mg/m^3) was only marginally significant with detection positive days decreasing with the amount of primary productivity (Fig. 6). Conversely, Hartel *et al.* (2015) found bottlenose dolphins in northern New Zealand to utilize deeper waters during the summer months and shallower waters in the winter months, and suggested that temperature associated with prey availability could be a possible factor explaining the difference in the fine-scale habitat use.

Even though the false positive rate reported with C-PODs is generally very low, between 1–4% (Nuuttila *et al.* 2013; Roberts & Read 2015), it is possible that some logged clicks were produced by other dolphin species than bottlenose dolphins. However, the likelihood of occurrence of this kind of false positives can be minimised by placing the devices in locations that are rarely, if ever, used by other species than the target species. Other dolphin species such as the common dolphin, *Delphinus delphis*, a relatively pelagic species (Perrin 2002; Bearzi *et al.* 2003), have never been observed in Killary Fjord, and their occurrence in McSwyne's Bay is rare; thus it is likely that the false positive rate due to other species is very low in this study. It has been shown that C-PODs are efficient at detecting echolocation bouts within a radius

of almost up to 1800m (Nuuttila *et al.* 2013). However, the GENENC click train classifier used in this study has, in general, a tendency for false negatives rather than false positives (Roberts and Read 2014; Robbins *et al.* 2015). This coupled with the fact that C-PODs use click trains to identify the occurrence of echolocation and thus will not detect events containing less than five successive clicks with similar inter-click intervals (Tregenza 2013), and that echolocation clicks are highly directional and off-axis click trains might be missed, may lead to an underestimation of dolphin occurrence. Nevertheless, due to their low false positive rate and efficacy in detecting click bouts, C-PODs can be considered as an efficient monitoring tool in predicting dolphin presence particularly in sites where habitat use is sporadic and unpredictable.

High mobility coupled with largely unknown ranging behaviour which can be driven by a suite of environmental or biological factors can present unprecedented challenges to the efficient monitoring of populations. This study demonstrates the potential of using passive acoustic monitoring devices, such as C-PODs, to unveil useful information on seasonal and temporal habitat use and can be used in conjunction with environmental factors to examine variables affecting the use of specific sites by bottlenose dolphins. The study also suggests that C-PODs can be used as a long-term monitoring tool in a relatively cost effective way that could potentially cover part of the management and monitoring requirements set by the EU Habitats Directive and the Marine Strategy Framework Directive.

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Chapter 5: Appendices

Appendix 5.1 Details of GAMs or GEE-GAMs ran on the C-POD data

	Model		
	Hourly	Daily	Monthly
Response variable	Presence/absence of click trains	Detection positive hours	Detection positive days
Distribution of response	Binomial	Poisson	Poisson
Covariates tested	Daylight (f)*, tidal level***, current speed***, current direction**	Site (f)**, year, Julian day, Site:Julian day interaction**	Temperature, productivity
Autocorrelation?	Yes → GEEs	Yes → GEEs	No
No. observations	24449	1021	25
No. independent blocks	154	154	25
Model predictive power	2%	8%	N/A

(f) denotes a factor variable
Significance levels *** <0.0001, ** <0.001, * <0.05

Chapter 6: Concluding discussion

Commonly used methods in conservation and management in the context of the present study

Protecting species and their habitats is the goal of conservation biology, and this could not be achieved without efficient management strategies. From the several methods currently applied to the assessment and management of cetacean populations, The International Union for the Conservation of Nature (IUCN) Red List Criteria classifies species to different categories on the basis of their abundance in relation to their risk of extinction: in short, a species with fewer than 50 mature individuals is classified as 'Critically Endangered', one with fewer than 250 classified as 'Endangered', and fewer than 1000 classified as 'Vulnerable' (IUCN 2016). In addition, species can be classified as of 'Least Concern' or 'Near Threatened' if they are not considered to be under immediate threat, and if sufficient data on their abundance exists (IUCN 2001). However, even though IUCN uses panels of experts to weigh the status of individual species against a Population Viability Analysis, it does not make any direct management recommendations (Lonergan 2011).

The European Union's Habitats Directive (Council Directive 92/43/EEC), on the other hand, requires each of the EU Member States to maintain or restore all the marine mammal species in European waters, at a 'favourable conservation status'. This status is reached when population dynamics data of a species indicate that populations are maintained at a viable level in the long-term in their 'natural habitat', the species' natural range is neither being reduced nor is likely to be reduced for the conceivable future, and a sufficient amount of habitat of suitable quality exists to maintain its populations on a long-term basis (Council Directive 92/43/EEC). Each Member State is required to report on the status within its boundaries every six years, however, it is up to the country to decide on the way it adheres to the requirements (as this is a Directive and not a Regulation). This approach has been criticised on the basis that the results of these reports are likely to be biased due to the lack of a common set of monitoring methods used in every EU Member State (Lonergan 2011). The Directive has further guidelines to defining the status of the species/populations and uses

'Favourable Reference Values' as baseline (Habitats Directive/Article 17); species that had a "normal" age structure and were above their "favourable reference population" were classed as 'Favourable'; those with a structure that "strongly deviated from normal", or were 25% below the "favourable reference population", or were below it and had declined by 1% annually in the previous six years were to be classified as 'Unfavourable – Bad', and all others 'Unfavourable – Inadequate'. However, one criticism raised for these guidelines has been that they do not consider the natural variation in population sizes when setting the initial 'Favourable Reference Values' (Lonergan 2011).

The EU Marine Strategy Framework Directive (MSFD, Council Directive 2008/56/EC) has the general goal to "achieve 'Good Environmental Status (GES)' of EU marine waters by 2020 and to protect the resource base upon which marine-related economic and social activities depend" (www.ec.europa.eu), thus it follows an ecosystem-based approach in the management of human activities whilst promoting sustainable use and protection of the environment. According to the MSFD, each EU Member State is obliged to develop an 'adaptive management strategy' for its marine waters, and this strategy needs to be reviewed every six years thus making the management protocols more dynamic and, at least, hopefully more responsive to any changes that may occur in population dynamics of protected species. In addition, the Member States are encouraged to co-operate regionally when developing marine strategies; the co-operation of different regions is coordinated through Regional Sea Conventions, for example in the case of Irish and UK waters, the relevant regional convention is the Oslo-Paris (OSPAR) Convention. The monitoring strategy within the MSFD developed by Ireland and involving the indicator species for GES (including bottlenose dolphins), is currently under review by OSPAR, but will be based on monitoring under the Habitats Directive (MSFD/Article 11). The MSFD's integrated approach towards ecosystem-based management is a welcomed idea along with its encouragement for co-operation between the Member States thus promoting the notion of network of SACs within EU waters. However, this approach will have to be backed up by clear and comprehensive management protocols. It remains to be seen how integrative these protocols, that are currently under development, will be.

Whereas the Habitats Directive (and possibly also MSFD) falls short in the sense that it does not provide specific guidelines to the Member States in the monitoring of the

status of species listed in Annex II and IV, in other jurisdictions, such as the USA, legislation such as the Marine Mammal Protection Act (1972) (MMPA) sets more rigorous and quantitative goals with the overall aim of preventing the depletion of local populations of marine mammals (referred to as stocks), and to restore them to level of Maximum Sustainable Yield (MSY). Later on, 'Potential Biological Removal' (PBR) was added to the Act (Wade 1998) as a way to quantitatively assess the effect of anthropogenic impacts on populations. The MMPA gives taxon/species specific guidelines regarding stock assessment (Wade *et al.* 1997), but in general, the purpose of the assessment is to determine the level of mortality that the stock can sustain. The calculations usually require several types of information, such as current and historical abundance, estimates of age of maturity, spatial distribution, rate of natural mortality, pregnancy rate (i.e. inter-birth interval), age distribution and MSY (Breiwick & York 2009). In addition to more comprehensive monitoring guidelines of the MMPA, the status of coastal bottlenose dolphin stocks is reported every 1–3 years in the US (http://www.nmfs.noaa.gov/pr/sars/species.htm); this is twice as often as required by the EU's Habitats Directive.

The main goals of this dissertation were:

1) to delineate the populations occurring in Irish waters as Management Units (MUs), to model the colonisation history of bottlenose dolphins in the wider North Atlantic Ocean with an emphasis on the colonisation of the coastal northern latitude habitats, and to describe and discuss some of the driving forces shaping the observed population structuring (Chapters 2 and 3)

2) to derive an abundance estimate for the wide-ranging 'coastal mobile' population and to estimate the scale of movements of this population (Chapter 4)

3) to describe the spatio-temporal variation in dolphin habitat use in the context of a number of environmental factors (Chapter 5), and

4) to discuss the findings in relation to the management of populations (Chapters 3, 4 and 5).

In Chapter 2 on phylogenetics and biogeography, the results indicated that the coastal bottlenose dolphins currently inhabiting the northern parts of the NE Atlantic may have

originated from refugial population(s) occupying the Mediterranean Sea during the Last Glacial Maximum via founder event(s), and that the colonisation of the NE Atlantic likely occurred following deglaciation after the LGM (see Fig 6.1.). This phylogenetic analysis includes, to the best of my knowledge, the first attempt to overcome the effects of time-dependency on the nucleotide substitution rate through a combination of using only third codon positions (sites where most mutations are silent, or synonomous, and are likely to be retained) of the coding genes of the mitochondrial genome and incorporating both tip calibration points from complete dated ancient mitogenomes and a fossil calibration on a deeper node. The estimates for nucleotide substitution rates, coalescence times and clade crown ages obtained in this study are in agreement with previously published estimates (e.g. Duchene *et al.* 2011; Moura *et al.* 2013; Morin *et al.* 2015), as well as the climatological and geological time frame, for example, the opening of the Bosphorous Strait and the retreat of the ice sheets covering Northern Europe.

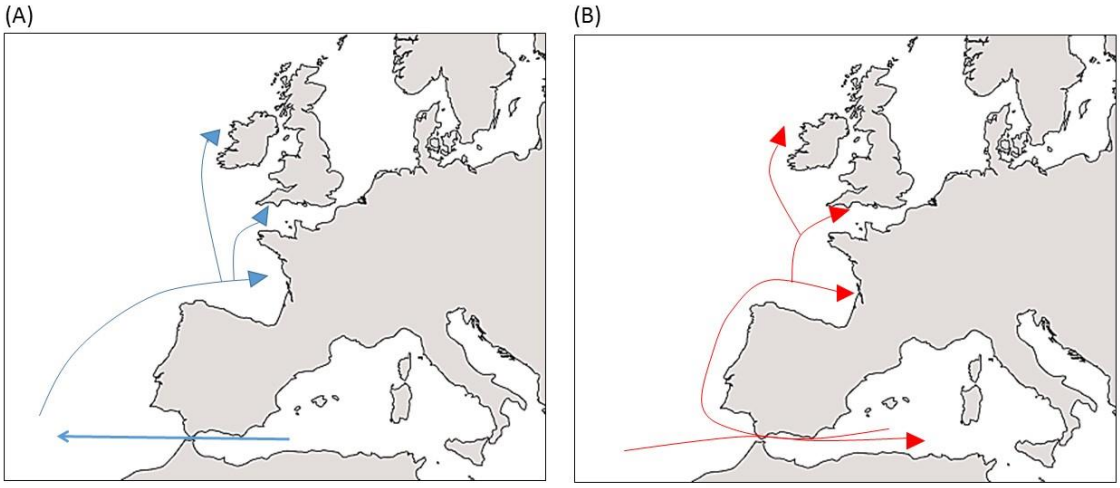

Figure 6.1 The most likely colonization patterns of bottlenose dolphins to the coastal NE Atlantic estimated in this study. (A) Clade consisting of samples from coastal West Ireland, West Scotland and Brittany. (B) Clade consisting of samples from coastal West Ireland, East Scotland, England, Wales and Brittany.

Climatic oscillations are thought to have played a role in shaping species distribution and divergence (Avise & Walker 1998), and combining population genetic data with models for suitable habitat such as the AquaMaps may offer a way to predict responses of current populations to the ongoing climate change, a field of research where only a

few examples exist (e.g. Inoue & Berg 2016). Data from previous genetic studies on marine mammals (Pastene *et al.* 2007; DeBruyn *et al.* 2009; Amaral *et al.* 2012; Foote *et al.* 2013) and other marine species (Crandall *et al.* 2012) have suggested that changes in population structuring and connectivity have followed glacial cycles. From this study, it also appears that cladogenesis events were correlated with periods of temperature change, with warmer temperatures at the onset of Holocene leading to an increase in sea-level in coastal areas and the subsequent release of available habitat coinciding with rapid radiation and population expansion of bottlenose dolphins into northern latitudes. These kind of rapid 'leading edge expansions' usually resulted in reduced genetic diversity in temperate species in large areas in the northern parts of Europe (Hewitt 1999) with a number of studies showing greater homozygosity in northern expansion areas (Hewitt 1996), and the northernmost coastal populations inhabiting the waters around Scotland and the west of Ireland are likely to be examples of this leading edge. In contrast, slower expansion and varied topography in southern European latitudes would, in general, retain more genetic diversity in southern populations allowing more time for divergence over many glacial periods in various southern refugia whereas northern temperate populations would die off during these colder periods (Hewitt 1999). This phenomenon is also documented in the current coastal bottlenose populations of the NE Atlantic, with the 'Coastal South' population having more genetic diversity in nuclear (Louis *et al.* 2013a) and mitochondrial markers (see Chapter 2).

The lack of differentiation of the 'Shannon' and 'mobile' bottlenose dolphin samples into separate haplogroups, or clades (Fig. 2.4, Chapter 2), indicates a recent population divergence that was found based on nuclear markers (Mirimin *et al.* 2011; Louis *et al.* 2014a, Chapter 3). In fact, Louis *et al.* (2014b) estimated the timing of divergence between 'Coastal South' and 'Coastal North' populations to have happened ~2560 yBP, and it is possible that the bottlenose dolphins resident in the Shannon estuary diverged from the 'mobile' population even more recently.

The work carried out for this PhD project has contributed to the assessment of the status of bottlenose dolphins in Irish waters. Specifically, in Chapter 3, further evidence was provided for the existence of three distinctive populations in Irish waters in support of Mirimin *et al.* (2011). This has been shown by analysing a larger set of genetic samples collected on a wider coastal area, and also by applying a range of statistical methods, including kinship-based methods and randomisation tests (e.g. Palsbøll *et al.* 2010). In addition, the photo-identification data in this study provided the first evidence for the near complete social isolation[1] of the two coastal populations, previously documented to exist only between the coastal and putative pelagic populations (Oudejans *et al.* 2015). For the first time, the recent (over the past two generations) genetic dispersal between the populations occurring in Irish waters was quantified, and it was established that the three populations are effectively genetically isolated and thus should be defined as separate Management Units (MUs) (see Chapter 1; Moritz 1994; Wade *et al.* 1997; Waples & Gaggiotti 2006; Palsbøll *et al.* 2006). Geographic isolation in new environments may contribute largely to divergence (Wright 1942). This is not likely to apply to the two populations of coastal bottlenose dolphins in Irish waters given their adjacent ranges, so the underlying reason behind the apparent reproductive isolation is likely to be driven by a combination of socio-ecological factors. More research effort is needed to fully elucidate the drivers of this existing population structure, but the evidence from this study infers that at least site-fidelity and social associations may be contributing factors. These reasons have been also suggested by several other authors to drive population structuring among several marine and terrestrial species (e.g. Parsons *et al.* 2006; Lowther *et al.* 2012; Louis 2014a,b; Podgórski *et al.* 2014; Gaspari *et al.* 2015). Long-term site-fidelity lasting over several years coupled with long and short term socials bonds and higher relatedness (compared to the offshore population, although this may be an artefact of smaller proportion of the offshore population being sampled) was found within the two coastal populations. Prey resources are temporally or spatially more predictable in coastal estuarine environments compared to open ocean habitats, and in these areas different foraging strategies may be socially and culturally transmitted (e.g. Mann & Sargeant 2003; Krützen *et al.* 2005) potentially leading to genetic divergence (see

[1] An individual sighted in McSwyne's Bay, Co. Donegal, in 2014 was photographed in the Shannon estuary in the summer of 2015. This dolphin was recorded associating with members of the Shannon community. However, the Shannon photo-id data from 2015 was not analysed for this thesis.

Kopps *et al.* 2014). However, some areas requiring more research effort were identified during the course of this study; for example, more tissue samples from 'pelagic' animals are needed in order to investigate whether further structuring exists within this population. Increased sampling of pelagic animals would also help to provide a more rigorous understanding of the distribution and habitat use of this population in contrast to the two coastal populations. In addition, in order to effectively assess sex-biased dispersal, more samples from females need to be collected from coastal populations, as biopsy sampling in Irish waters so far has been concentrated largely on males possibly due to their distinctive and more obvious markings. Nevertheless, from a management point of view, the fact that these coastal bottlenose dolphins showed non-overlapping ranges with site-fidelity to each of the coastal SACs, implies that the designation of these areas to correspond with the different populations has been successful.

Following the delineation of MUs, the abundance of the 'coastal mobile' population was estimated in Chapter 4 using a relatively novel Bayesian multi-site method in mark-recapture abundance estimation. Bayesian methods have especially been applied to abundance estimation of cetacean species (e.g. Durban et al 2005; Durban *et al.* 2010; Moore & Barlow 2011; Fearnbach *et al.* 2012; Cheney *et al.* 2013) as they are well-suited for sparse data sets. The Bayesian multi-site approach is particularly useful when it is not feasible to apply a survey strategy covering the entire distribution of the population in question (Durban *et al.* 2005). This method can be used relatively economically with the help of a sightings network when the presence of the animals is unpredictable and spatially patchy and the surveys are largely limited by budget and weather conditions. By using this multi-site approach, where re-captures of individuals were ordered on a spatial rather than temporal scale, an abundance estimate was derived that is both accurate and robust for the wide ranging 'coastal mobile' population of bottlenose dolphins that use the West Connacht Coast SAC. Deriving an estimate using only data collected within the designated area would have likely resulted in an underestimation of abundance due to the potential failure to capture some of these highly mobile animals. In addition to abundance estimation, it was determined that six consecutive years of annual monitoring would be required to detect a 10% annual decline in population, even with a low CV of 0.11. Therefore, it is recommended that monitoring should be continued on a yearly basis. Moreover, in

order to be able to detect an overall decline of 25% in abundance over a six-year reporting period, a limit set by the Habitats Directive, the CV around the abundance estimate would have to be as low as 0.08, which could be difficult, if not impossible, to achieve for a mobile population such as the one in this study. As a conclusion, continued and more wide-scale research effort is recommended in order to detect any changes in population dynamics within the coastal populations, and to better understand their ranges. Shorter reporting interval of 1–3 years used in the management of the coastal bottlenose dolphin 'stocks' in the US, combined with a thorough assessment of mortality and fecundity rates, should be also applied to the management of coastal populations within European waters.

The Bayesian multi-site method offers great potential to be used as a monitoring tool for networks of MPAs, such as the national SACs that form part of a European-wide conservation instrument directed to protect populations on a wider transboundary level. More international co-operation, encouraged by the MSFD and to some extent also the Habitats Directive, is required in the assessment of the status of the coastal mobile bottlenose dolphin population, especially given that evidence suggests that these mobile animals have a range extending beyond country boundaries (Robinson *et al.* 2012), and that they may belong to a wider meta-population of dolphins occupying the waters of Ireland, Scotland and northern France (Nichols *et al.* 2007; Louis *et al.* 2014a). In fact, plans for a multi-national project to assess the population status of bottlenose dolphins in countries of the "Atlantic Arc" (Ireland, UK, France, Portugal and Spain) are under way. In the meantime, a smaller scale project resolving the (meta)population status of the bottlenose dolphins in Irish waters could be done by analysing a common set of microsatellite markers used by Louis *et al.* (2014a) or the SNP markers generated in a recent study (see Louis, Nykänen *et al.* in prep, Appendix 6.1).

Studying how individuals use areas on a smaller spatial scale is a question that also poses logistical difficulties and is often confined to summer months in northern latitudes due to weather conditions alone. For this thesis, site occupancy of bottlenose dolphins within a location inside the West Connacht Coast SAC was examined using passive acoustic monitoring (Chapter 5), and compared to site occupancy in another location over 100km north outside the SAC boundaries. The effect of environmental factors that are likely to influence habitat use of bottlenose dolphins were also

examined. Killary Fjord, Co. Galway, was identified as an important site for the dolphins with monthly detections and significantly more detection positive days logged in this site compared to McSwyne's Bay, Co. Donegal. This indicates that the area designated as a bottlenose dolphin SAC containing the area around Killary Fjord is indeed a site regularly used by dolphins. A peak in detections occurred in the spring in this site which may be a response to a local increase in prey availability. These results show that assessing the effectiveness of SACs can be supplemented by PAM, and it is recommended that monitoring be continued in order to gather long-term evidence of the effect of seasonal trends in relation to dolphin occupancy, as only 13 months of continuous data from both sites during this study were collected. In addition, more C-PODs should ideally be deployed in areas within the SAC as well as outside, in order to identify other areas of importance and seasonal factors effecting site occupancy. Moreover, having a strategically placed network of C-PODs along the coast of Ireland could increase the likelihood of detecting changes in the site occupancy and help to re-evaluate the status of the current SAC.

Areas for future research

In general, this thesis demonstrates that applying a combination of methodological approaches and selecting these approaches to suit the populations in question can provide an efficient way to monitor mobile populations with unpredictable ranges and habitat use such as the transient population of bottlenose dolphins on the west coast of Ireland. However, some areas in which further research is required were identified are discussed below.

Understanding the spatial distribution of organisms needing protection is one of the key aspects for effective management and conservation (Whittaker *et al.* 2005). The full ranging patterns of these highly mobile animals (i.e. the 'coastal mobile' population) is not known; however, evidence exists that at least some identified individuals range widely and beyond trans-national boundaries. One solution to overcome this challenge caused by high mobility and unpredictable movement patterns could be to increase the collaboration between researchers in different countries and areas. This would include sharing of the photo-id catalogues and tissue samples between research institutes. Repeated wide-scale survey effort is essential in

order to be able to detect shifts in distribution of these animals and also to detect changes in their abundance. Another approach to investigating animals' ranging behaviour would be to conduct a telemetry study using remotely implanted tags attached to individual dolphins. Satellite tagging has the advantage of providing continuous movement and range information over the period the tag is attached and transmits data. A disadvantage is that it would only be possible to tag a few individuals due to the cost and the logistical difficulties involved in the tagging. However, this approach would be highly complementary when combined with photo-id and genetic mark-recapture methods. At present there are ethical considerations for such a tagging programme, but tags are becoming smaller and more readily employed using remote methods such as rifles and crossbows (e.g. LIMPET-tags, Wildlife Computers Inc.) instead of more conventional capture tagging methods.

More effort should also be invested into estimation of demographic parameters such as survival, mortality rate, the calving rate and the proportion of adults/subadults in the population. Most higher predators have long life spans, and consequently it can take several years before any changes in population growth or structure become apparent; again this emphasises the need for regular monitoring. A potentially promising method in detecting changes in fecundity and survival within a population would be to look at changes in age structure (Holmes & York 2003). Recently, it was found that fluctuations in reproductive rates can have considerable impacts on population viability of bottlenose dolphins (Manlik *et al.* 2016), thus again highlighting the importance of consistent monitoring of these parameters. Consequently, models of population viability could be run including different population parameters, such as the ones listed above and following Manlik *et al.* (2016), and with different levels of prey resources (e.g. depleted fish stocks).

Other future research could also include comparison of genomic differentiation and gene expression between the two distinct coastal populations, to identify functional regions, and to subsequently investigate the role of different genes in local adaptation (see review by Kelley *et al.* 2016). In addition, greater geographic resolution in resolving NE Atlantic bottlenose dolphin phylogeny could be obtained by performing a larger scale analysis with a wider coverage of samples from the pelagic and other coastal northern European populations. In fact, twenty more modern samples collected from the coastal areas of Scotland and three radio-carbon dated ancient subfossil

samples originating in the Dutch Southern Bight in the North Sea will be sequenced in the near future and analysed with the dataset presented in Chapter 2.

A comprehensive estimate of diet combined with genetic sampling is required to investigate the role of resource partitioning as a driver of species or population structure (e.g. Foote *et al.* 2009; Kiszka *et al.* 2011; Ryan *et al.* 2013; Ansmann *et al.* 2014; Louis *et al.* 2014b). Hernandez-Milian *et al.* (2015) examined the stomachs of 12 bottlenose dolphins stranded around the Irish coast, but these samples have not all been genotyped. The investigation of stable isotopic signatures has the potential to reveal differences related to population structure on a wider scale, for example, the coastal – offshore separation (e.g. Louis *et al.* 2014b; Rogan *et al.* in prep.) or sympatric species or populations with overlapping distributions (e.g. Foote *et al.* 2009; Kiszka *et al.* 2011; Ryan *et al.* 2013; Ansmann *et al.* 2014).

Utility of Marine Protected Areas in the conservation of marine mammals

According to the definition by the World Conservation Union (IUCN 1994), a Marine Protected Area (MPA) is "any area of intertidal or subtidal terrain, together with its overlying water and associated flora, fauna, historical and cultural features, which has been reserved by law or other effective means to protect part or all of the enclosed environment". MPAs have recently evoked discussion on their usefulness in preserving biodiversity or protecting a specific species or population (e.g. Hooker & Gerber 2004; Agardy *et al.* 2011; Hartel *et al.* 2015; Wilson 2016). One criticism has been the argument that the establishment of MPAs is sometimes solely driven by the interest shown by the public to charismatic species like marine mammals (Hooker & Gerber 2004; Wilson 2016), and they have sometimes been referred to as 'paper parks' that lack the necessary monitoring and regulation (Duffus & Dearden 1995; Hooker *et al.* 1999; Agardy *et al.* 2011). One example of this would be the designation of so called whale sanctuaries that extend to cover the entire EEZs of countries (Rogan & Berrow 1995; Hoyt 2005; Agardy *et al.* 2011). On first thought this idea seems very appealing with the general aim of promoting the protection of marine mammals, however, the question remains whether it is appropriate to call an area truly protected when these designations are not accompanied by risk assessment, mitigation and a set of specific management actions.

Marine Protected Areas have also been criticised for having been designated in areas that hold less importance for the species under protection whilst leaving more important areas unprotected due to various political or economic reasons. For example, the Pelagos Sanctuary in the Mediterranean Sea covering areas in waters of Italy, France and Monaco provides protection to areas that are of relatively low value for marine mammals whilst leaving out more important areas due to potential difficulties in managing these areas (Agardy *et al.* 2011). When protection is directed to a specific population, the ideal situation would be to ensure protection over the entire population's range which it inhabits year-round (Reeves 2000). However, some mobile marine predators, such as the sperm whale, have a global population structure possibly through male-mediated gene flow and a distribution that extends from feeding grounds in low latitudes to calving area in high latitudes (Lyrholm & Gyllensten 1998; Alexander *et al.* 2016). The designation of an MPA to cover such a large offshore area would not be economically or politically feasible in most parts of the world. Thus one of the criticisms towards MPAs has been that they are too small and represent only a minute portion of the total range of the species (Hooker & Gerber 2004; Agardy *et al.* 2011; Wilson 2016). As an example, the generally applied criteria used in the designation of MPAs for marine mammals has primarily been concentrated on preserving breeding areas without taking to account foraging habitats or migration routes (Hooker & Gerber 2004). Yet, it is likely that most marine mammals are at most risk while foraging (e.g. harbour porpoise, ASCOBANS 2012). However, Hooker and Gerber (2004) also argued for the general benefits of MPAs that are designated based around vulnerable life stages, such as breeding/calving areas, by stating that even if the area were used by a species only for a portion of its life span, this would still diminish the overall cumulative impact of other threats, thus reducing the frequency with which each individual was exposed (Hooker & Gerber 2004). The bottlenose dolphin SACs in Irish coastal waters have been designated based on core ranges estimated from encounters during photo-identification surveys and considering the two distinct populations. However, McSwyne's Bay was identified as an important site for bottlenose dolphins during previous surveys (Ingram *et al.* 2001) and this study, suggesting that this area should be considered for further SAC designation.

A considerable amount of thought has to be put into the management of MPAs. For example, managers are often too concerned about managing an MPA alone without

paying attention to the management of the entire population. Concentrating all monitoring efforts on a designated area that covers only a part of a mobile and wide ranging species, could give a biased view of the status of the population, if its range has shifted to other areas (e.g. Wilson *et al.* 2004). An economic solution to overcome this problem could be the deployment of a network of passive acoustic monitors to monitor the site use within the protected area and the wider area around it. Other methods could include the use of novel multi-site methods in abundance estimation that incorporate estimates on the scale of movement of animals within and outside of a MPA. Both of these methods were used in this study.

There are, however, some positives in amongst all the criticism that MPAs have received. For example, while most existing protected areas are isolated and thus connectivity between sites is not ensured, the Great Barrier Reef Marine Park in Australia has been described as a success story of a large scale network of MPAs with its integrated and adaptive management (e.g. McCook *et al.* 2010). Even when a MPA is designated to cover a relatively small isolated area, it can be successful, as in the case of the Banks Peninsula Marine Mammal Sanctuary designated for Hector's dolphins (*Cepahlorhychus hectorii*). A recent study reported a 5.4% increase in mean survival probability following the designation of the MPA accompanied with a ban on commercial and a restriction in amateur gillnetting in the area (Gormley *et al.* 2012). The Natura 2000 network of SACs for bottlenose dolphins at the European scale seems to be a step to the right direction because a network of SACs may ensure the protection of areas important for the dolphins and enhance connectivity between the SACs. However, transnational co-operation in the monitoring of these areas is required since the Member States are responsible for reporting on the status of species only in their own national SACs, and populations can have ranges extending beyond country boundaries. Hopefully the OSPAR convention will provide a solution by producing a comprehensive and effective management protocol that the Member States will then adhere to.

Static MPA boundaries may not be the most appropriate method to manage marine mammals. More dynamic MPAs where the boundaries can be adjusted in response to changing species distributions or site use have been suggested by several authors (Hooker & Gerber 2004; Hooker *et al.* 2011; Hartel *et al.* 2015). How this will be accepted by managers is, however, uncertain since the practicalities involved in

shifting the boundaries and management of these dynamic areas may turn out to be logistically problematic. Another approach suggested by several authors has been the development of more comprehensive marine spatial planning and ecosystem based management strategies (e.g. McLeod *et al.* 2005; Halpern *et al.* 2009; Agardy *et al.* 2011; Wilson *et al.* 2016) that emphasise the protection of the ecosystem as a whole whilst acknowledging the connectivity among systems (McLeod *et al.* 2005). The European Union's Marine Framework Directive is an example of this (with bottlenose dolphins being listed as one of the indicator species of good environmental status of coastal habitats), but it remains to be seen how the management will be applied to these habitats in order to ensure the protection of the populations.

In conclusion, it is positive and forward thinking that the different populations with their non-overlapping core ranges have been taken into consideration in the designation of the SACs in Irish coastal waters, even though they do not cover the entire (largely unknown) ranges of the populations. Based on the results of the photo-id and acoustic monitoring work in this study, it seems that the areas around Killary Fjord are important for the dolphins. However, the area in Donegal Bay outside the SAC boundaries also seems important based on the regular encounters of large groups consisting of up to 100 dolphins (see Chapter 4). More research effort will be required to uncover the distribution of these animals with the possibility of extending the existing SACs to cover areas further north. These areas should at least cover the core ranges of the dolphins. Efficient and regular monitoring of the populations should be continued so that any changes in population parameters can be detected and the best possible conservation strategies implemented in a timely manner, ensuring the long-term viability of the populations.

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Chapter 6: Appendices

Appendix 6.1. Manuscript of SNP discovery (*in prep.*)

High density, genome-wide SNP discovery in Northeast Atlantic bottlenose dolphins based on genotype likelihoods from multiplex shotgun sequencing data

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Abstract

The discovery and development of a high density, genome-wide SNP array can be an important step towards a thorough understanding of local adaptation, mutation load and demographic history and can therefore inform the conservation and management of species. Bottlenose dolphins, *Tursiops truncatus*, are protected by the Habitats Directive in European waters where they form large offshore and small localized coastal populations. Here, we present a simple and relatively low-cost approach to SNP discovery by shotgun sequencing in this species. We shotgun-sequenced indexed libraries of 33 individuals from the North-East Atlantic. As the mean coverage across sites was low (mean coverage of 4×), we identified SNPs by estimating genotype likelihoods. We then applied a series of stringent filtering steps to remove SNPs in potential repeat regions, paralogous regions, NUMTs and regions of low mappability. This resulted in the discovery of a total of 440,718 SNPs. We validated 266,187 SNPs by comparing our data to two published high coverage (>30×) full genomes of bottlenose dolphins, one originating from the Pacific, and the other from the North-West Atlantic. Whilst this approach only results in SNP discovery rather than simultaneous SNP discovery and genotyping, it only needs to be done once for a set of populations, allowing subsequent studies to target sequence this reference set of SNPs using hybridization-enrichment capture for a broad range of applications (i.e. inferences of selection and demographic history). We highlight how this resource could be used to optimize the number of SNPs targeted in different RAD-seq strategies by simulating RAD-seq experiments with *in silico* endonuclease cutting sites.

Introduction

The advent of high-throughput sequencing technologies coupled with the development of population genomic approaches have facilitated the use of genome-wide data for the inference of local adaptation, historical demography and admixture. In particular, RAD-sequencing (RAD-seq), genotyping-by-sequencing (GBS), and associated reduced representation library (RRL) methods (Baird *et al.* 2008; Davey *et al.* 2011; Elshire *et al.* 2011; Davey *et al.* 2013; Andrews *et al.* 2016) allow relatively low-cost simultaneous SNP (Single Nucleotide Polymorphism) discovery and genotyping of large numbers of individuals, and have therefore become increasingly more widely used in empirical studies (see Narum *et al.* 2013 for a review). RAD sequencing has generated a large number of SNPs in some species such as the European eel (*Anguilla anguilla*) (376,918 SNPs, Pujolar *et al.* 2013) and rainbow trout (*Oncorhynchus mykiss*) (145,168 SNPs, Palti *et al.* 2014). However, these reduced representations of the genome might not be well suited for some applications for species with low genetic diversity without optimization to increase the number of SNPs targeted. As an example, cetaceans typically have slow molecular clocks (Bininda-Emonds 2007; Jackson *et al.* 2009; Dornburg *et al.* 2012; McGowen *et al.* 2012) and low nucleotide diversity (Table 1, Yim *et al.* 2014). Consequently a relatively low number of SNPs (typically <10,000) have been discovered by RAD-sequencing studies in this taxonomic group (Moura *et al.* 2014a; Viricel *et al.* 2014; Cammen *et al.* 2015; but see Fernández *et al.* 2016, Table 2). Given the low genetic diversity in many cetacean species, RRL data may not be ideal for some inferences of intra-specific population history. For example, the accuracy of demographic inference based on the site frequency spectrum (e.g. Gutenkunst *et al.* 2009; Excoffier *et al.* 2013; Liu & Fu 2015) is a function of the number of segregating sites (Terhorst & Song 2015). Furthermore, many inferences of intraspecific history including demographic history and selection utilize information from multiple linked polymorphic sites spanning longer contiguous sequences (e.g. Nielsen *et al.* 2005; Li & Durbin 2011). Therefore, for those particular applications in species with low genetic diversity yielding low density of SNPs within RRLs, either an alternative approach and/or optimization of the RRL method to maximize SNP yield may be needed.

Here, we use an alternative, simple and low cost approach to SNP discovery by shotgun sequencing (*SDBSS*) in the bottlenose dolphin (*Tursiops truncatus*), a species with relatively low genetic diversity (Table 1, Yim *et al.* 2014). Previous studies have identified 153 SNPs by sequencing targeted regions of the genome and 7,431 SNPs using RAD-sequencing in this species (Vollmer & Rosel 2012; Cammen *et al.* 2015, Table 2). Bottlenose dolphins are long-lived, social marine mammals that have a worldwide distribution. Their range includes a large variety of habitats including temperate and tropical, coastal, deep pelagic and insular waters. Their ecology and morphology are highly variable across their range, with two ecotypes "pelagic" and "coastal" reported in the North-West Atlantic (NWA), North-East Pacific (NEP) and North-East Atlantic (NEA) (Hoelzel *et al.* 1998; Segura *et al.* 2006; Louis *et al.* 2014a; Louis *et al.* 2014b; Lowther-Thieleking *et al.* 2015). In the NEA, coastal populations have likely been founded by the pelagic population relatively recently, after the Last Glacial Maxima (10,320 yrBP, 95% CI: 4,300–47,800, Louis *et al.* 2014a). The most likely hypothesis is that they originated from pelagic individuals that colonized European coastal waters when the sea ice retreated. Therefore, they are an interesting study system to investigate the influence of both adaptive and demographic processes during genomic divergence. As detailed previously, these analyses require the development of a large set of genetic markers. In addition, bottlenose dolphins are protected in European waters by the Habitats Directive (Council Directive 92/43/EEC) and the conservation of the species requires a thorough understanding of local adaptations, population structure, demographic history and gene flow. Efforts to compare among genetic studies focusing on localized populations have so far been hindered by the use of different microsatellite markers. The development of a SNP resource will overcome these comparison issues, allowing future studies to target the same markers and provide a complete understanding of population structure. Moreover, studies on this species can benefit from the availability of two full high coverage (>35×) genomes: a US Navy dolphin from the Gulf of Mexico (Sam Ridgway, personal communication); and a dolphin from the Pacific (Lindblad-Toh *et al.* 2011; Yim *et al.* 2014; Foote *et al.* 2015). Given the recent divergence of bottlenose dolphins in the NEA (Moura *et al.* 2013; Louis *et al.* 2014a) and the slow molecular clock of cetaceans (Bininda-Emonds 2007; Jackson *et al.* 2009; Dornburg *et al.* 2012; McGowen *et al.* 2012), many polymorphic sites are likely to be standing genetic variants shared globally across the populations throughout the species' range and these

high coverage genomes are therefore anticipated to be able to validate many of our inferred SNPs.

For this study, we mined whole genome shotgun-sequencing data that included both coastal and pelagic bottlenose dolphins from the NEA, to investigate the potential for such data to be used for the discovery of SNPs in the nuclear genome. We applied stringent filtering steps to limit errors linked to sequencing and low-coverage data. In order to identify putative global variants, we compared our data to the two available bottlenose dolphin full genomes. In addition, we checked our data against the two full genomes of another delphinid species, the killer whale (*Orcinus orca*) (Moura *et al.* 2014b; Foote *et al.* 2015), to identify SNPs that correspond to potential ancient standing variants within the Delphinidae. We provide a large SNP resource that could be genotyped using hybridization-enrichment capture methods for downstream population genomics analyses of NEA bottlenose dolphins. Users could also use this SNP dataset to customise RAD-seq strategies to optimize the number of targeted SNPs.

Materials and methods

DNA extraction, library preparation and sequencing

Thirty-three samples of epidermal tissue samples were collected from stranded and biopsied sampled free-ranging individuals (see Louis *et al.* 2014b; Nykänen *et al. in preparation*) from France (N = 12), England (N = 2), Ireland (N = 9), Wales (N = 3) and Scotland (N = 7, Figure 1 plotted using the MARMAP package (Pante & Simon-Bouhet 2013) in R 3.2.0 (R Core Team 2015)). The samples were previously genotyped using microsatellites and genetically assigned (apart from 1 individual) to coastal (N = 21) or putative pelagic (N = 11) populations using population genetics methods such as STRUCTURE, TESS and DAPC (Islas 2010; Mirimin *et al.* 2011; Louis *et al.* 2014b). DNA was extracted from epidermis tissue using a Qiagen DNeasy kit following the manufacturer's guidelines. Genomic DNA was then sheared to an average size of ~150-200 bp using a Diagenode Bioruptor NGS run with 20 cycles of 30 seconds on, and 30 seconds off. Illumina sequencing libraries were built on the sheared DNA extracts using NEBNext (Ipswich, MA, USA) DNA Sample Prep Master Mix Set 1 following Meyer and Kircher (2010). Libraries were subsequently index

amplified for 15 cycles using Phusion High-Fidelity Master Mix (Finnzymes) in 50-μL reactions following the manufacturer guidelines. The libraries were then purified using a MinElute PCR purification kit (Qiagen, Hilden, Germany). The DNA concentration of the libraries was measured using a 2100 Bioanalyzer (Agilent Technologies, CA, USA), these were pooled approximately equimolarly and then sequenced on an Illumina HiSeq 2500 platform as a lane of 100-bp single read (SR) using v4 chemistry.

Base calling, sequence read trimming and mapping

Conversion of Illumina's *.bcl files to fastq, and demultiplexing were performed using Illumina's CASAVA 1.8.2 software allowing for no mismatch in the 6-nucleotide indices used for barcoding. Sequencing reads within the generated fastq files were processed with ADAPTER-REMOVAL (Lindgreen 2012) to trim residual adapter sequence contamination and to remove adapter dimer sequences as well as low-quality stretches at 3´ ends (i.e. consecutive stretches of N's and of bases with a quality score of 2 or lower). Sequence reads that were ≤30 bp following trimming were discarded. The remaining filtered reads were first mapped to a bottlenose dolphin mitochondrial genome to be removed and analyzed separately. Reads that did not map to the mitochondrial genome were then extracted from the bam file and converted into a fastq file using SAMTOOLS (Li *et al.* 2009). These reads were then mapped, requiring a mapping quality greater than 30, to the reference bottlenose dolphin genome assembly (Ttru_1.4/turTru2, GenBank Assembly ID: GCA_000151865.2, Lindblad-Toh *et al.* 2011; Foote *et al.* 2015) using BWA (v. 0.6.1) (Li & Durbin 2009), which had been hard-masked using REPEATMASKER (Smit *et al.* 1996) and TANDEM REPEATS FINDER (Benson 1999) and was accessed from the UCSC genome browser (Karolchik *et al.* 2014). Clonal reads were collapsed using the rmdup function of the SAMTOOLS (v. 1.2.1) suite (Li *et al.* 2009). Short read data from two bottlenose dolphins and two killer whales *Orcinus orca* (PRJNA20367, PRJNA167475, SRR940825, Lindblad-Toh *et al.* 2011; Moura *et al.* 2014b; Yim *et al.* 2014; Foote *et al.* 2015) were additionally accessed from the National Center for Biotechnology Information Sequence Read Archive database and mapped to the reference genome assembly as above. Coverage was then estimated for each genome using the doDepth function in the ANGSD software package (Korneliussen *et al.* 2014).

Multi-sample genotype likelihood calling

Our SNP discovery pipeline followed the suggested best practices outlined in Nielsen *et al.* (2011). Briefly, we used a multi-sample SNP calling approach, taking uncertainty into account by calculating genotype likelihoods, as recommended by Nielsen *et al.* (2011). Uncertainty in SNP calling in low coverage data (i.e. sequencing depth <6×) can arise from sequencing, base-calling, mapping and alignment errors. To limit these biases, the quality scores of the sequencing data can be integrated in probabilistic methods to calculate genotype likelihoods. The genotype likelihood is the marginal probability of the read data given the genotype of a particular individual at a particular site that is rescaled by the quality score of each read (Nielsen *et al.* 2011). In addition, variant discovery accuracy and efficiency with low coverage data are largely improved using multiple samples in comparison with a single sample (Nielsen *et al.* 2011). First, the reads may have only been sampled on one of the two chromosomes of a diploid individual. Thus, calling SNPs from only one sample or after combining the results from each individual separately would lead to very low power (Li 2011). In addition, multiple samples allow the discovery of SNPs based on the estimated allele frequencies (Nielsen *et al.* 2011). Sites were called as SNPs if the minor allele frequency was significantly different from 0 as inferred from a likelihood ratio test using a chi-square distribution with one degree of freedom (Kim *et al.* 2011). We set a conservative threshold of only calling SNPs at sites inferred to be variable with a probability of $P < 0.000001$ by the likelihood ratio test. We calculated the genotype likelihood using the SAMTOOLS method implemented in ANGSD (Li 2011; Korneliussen *et al.* 2014) based on an Expectation Maximization algorithm to both infer the major and minor alleles and to estimate major and minor allele frequencies (Kim *et al.* 2011; Skotte *et al.* 2012). The major allele was inferred and uncertainty in the determination of the minor allele was taken into account by summing over the three possible alleles weighted by their probability (Kim *et al.* 2011). The identified SNPs could be due to heterozygous sites within individuals, or alternative alleles being sequenced in different individuals. SNPs were called separately for the NEA bottlenose dolphin shotgun sequencing (SGS) data, the two high coverage bottlenose dolphin genomes and the two high coverage killer whales.

SNPs filtering

Five filtering steps were then applied to the SNPs inferred from the genotype likelihood estimation using R 3.2.0 (R Core Team 2015) to further avoid artifacts linked to NGS and low-coverage data. We first filtered SNPs in regions of poor mapping quality (Q<30). Then, we discarded SNPs with a depth of coverage higher than twice the mean coverage (mean coverage is 5×). High coverage of these SNPs is potentially the result of unmasked repeated regions, in nuclear mitochondrial DNA (NUMTs), or some other mapping artifact (e.g. paralogous loci). Regions of poor mapping quality (Q<30) and excessive coverage (>10×) were detected using the CALLABLELOCI tool in GATK (McKenna *et al.* 2010; DePristo *et al.* 2011). As a further step to remove regions of excessive coverage which could have arisen due to the mapping issues we highlighted above, we plotted the number of SNPs against the number of individuals and discarded the SNPs in the upper tail of the distributions: specifically SNPs found in more than 10 individuals. As it can be difficult to accurately call variants with MAF < 0.1 (Maruki & Lynch 2015), we further discarded the SNPs with estimated minor allele frequency (MAF) < 0.1. Nevertheless, estimations of genotype likelihoods rather than genotype calling are expected to substantially reduce the error rate. In addition, rare variants are useful for many applications such as for the inference of demographic history based on the SFS and the estimation of several population genetic parameters such as diversity and F_{ST} estimates (Nielsen 2004; Clark *et al.* 2005). Therefore, the data from this filtering step are retained in the dryad depository.

Each of these four filters was applied to the original SNP set inferred from the genotype likelihood estimates to evaluate the number of kept and discarded SNPs at each step. The extent of overlap in the SNPs removed by the different filtering steps was visualized using a Venn diagram. The objective of this study was to develop a SNP array for NEA bottlenose dolphins, thus, all samples were from the NEA. Therefore, our SNP dataset could be subject to ascertainment bias if used for some population genetics inferences on samples from another geographical area (Nielsen 2004). To define a set of putative global variants for this species, we identified SNPs that were also polymorphic or had alternative alleles in either one or both high coverage sequences of individuals originating from the Pacific and the West Atlantic. All the filtering steps were then applied to the original SNP set to get a final set of high

confidence global variants. Lastly, to identify ancestral polymorphisms within the Delphinidae, we repeated the above, identifying sites that were polymorphic across the bottlenose dolphins and the killer whales.

Population genomic exploration of SNP efficacy

NGSADMIX (Skotte *et al.* 2013) was used to estimate individual's ancestry based on genotype likelihoods of the filtered total NEA SNPs (i.e. SNPs that passed the first four filtering steps but that were not filtered based on shared polymorphism with the two high coverage genomes), therefore avoiding inferring individual genotypes and taking the uncertainty in genotype calling into account. NGSADMIX is a maximum-likelihood based clustering method that can provide reliable population structure results with very low coverage data as shown by simulations and real data analyses (Skotte *et al.* 2013). We acknowledge that our dataset is sparse and contains missing data, and thus population structure inferences should be only considered as exploratory, with the aim of providing some indication that the SNP array is informative (i.e. can detect population structure). In addition, NGSADMIX assumes that the loci are at Hardy-Weinberg Equilibrium (HWE) and we acknowledge that given the coverage of the data we could not test for this. NGSADMIX was run for a number of ancestral populations with *K* set from 2 to 4 and including all SNPs, polymorphic sites found at sites covered in at least 5 individuals and at least 9 individuals with 3 replicates runs. Individuals' ancestry proportions were compared to previous microsatellite based studies (Islas 2010; Mirimin *et al.* 2011; Louis *et al.* 2014b). The best number of ancestral populations *K* was inferred based on the known population structure inferred in these microsatellite studies.

In silico endonuclease digestion experiments to customize and optimize RAD-seq strategies

We performed *in silico* cut experiments to evaluate the number of SNPs generated by different RAD-sequencing strategies using a script which we have provided in the dryad depository. Using the bottlenose dolphin reference genome, RAD data were simulated by sampling sequences proximal to the restriction sites. Different parameters were tested including the enzymes used (*NotI*, *SbtI* and a combination of *NotI* and *SbtI*, *i.e. double digest* RAD-seq) and the fragment size selection (minimum and maximum

length of the fragment). We then evaluated the number of total filtered NEA SNPs generated by each RAD-seq strategy.

Estimates of the scaled mutation rate, θ from whole genome sequences of several cetacean species

Whole genome sequence reads of single individuals of several cetacean species from Yim *et al.* (2014), Foote *et al.* (2015) and Keane *et al.* (2015) were extracted from the National Center for Biotechnology Information Sequence Read Archive database and assembled as detailed previously. Maximum likelihood estimates of θ were computed from the assembled reads using mlRho version 2.8 (Haubold *et al.* 2010, 2014).

Results

Estimates of the scale mutation rate, θ from whole genome sequences of several cetacean species

Diversity estimates were relatively low in all cetacean species (Table 1). Bottlenose dolphins showed intermediate θ estimates in comparison to other cetacean species (Table 1).

Raw data

We generated 292×10^6 sequencing reads from our shotgun sequencing data of 33 individuals, of which 205×10^6 uniquely mapped to the reference genome. We retained bases with a minimum Phred score of Q30 (Figure 2a illustrates the distribution of the quality scores). The mapped data from all individuals resulted in a mean of 5× coverage of the genome, but the sequencing reads for each individual covered only a fraction of the genome, with few bases being covered >1× by reads from the same individual (Figure 2b). For 28 of the 33 NEA individuals, >1 million reads were generated for each individual (Supplementary Table 2).

Identification of high likelihood polymorphic sites

The likelihood ratio test inferred 530,844 sites to be polymorphic at a probability of $P < 0.000001$ in the NEA bottlenose dolphin SGS data. We further identified 4,466,188 and 1,022,488 SNPs in the two high coverage bottlenose dolphin genomes and the two high coverage killer whale genomes, respectively. After filtering out SNPs (Figure 3a) with poor mapping quality (Q<30), sites with excessive coverage (>10×, Figure 3b),

SNPs sequenced in >10 individuals (Figure 3c), SNPs with a MAF < 0.1 (Figure 3d) and SNPs that were not polymorphic in the two dolphin high coverage genomes, 266,187 global variants were retained. A further set of 174,531 SNPs that passed the first four filtering steps but were only found in the NEA dolphins could be useful for population genomic studies in the NEA region. They may however be prone to ascertainment bias when genotyped in other geographical areas (see discussion). The number of SNPs that were kept and removed by each filtering step is given in Table 3 and lists of the positions of SNPs that were kept after each filtering step are provided in dryad. Relatively high numbers of SNPs were simultaneously removed by the coverage, number of individuals and/or mapping quality filters suggesting that they were unmasked repeats or some other artifact (Figure 4). A total of 33,489 SNPs were shared between the filtered NEA dolphin variants and the two high coverage killer whale sequences, and 169,016 SNPs were also polymorphic across the two high coverage bottlenose dolphins and the two killer whale sequences. 30,932 SNPs were shared between the three datasets. SNP sharing is visualized in a Venn diagram drawn using the online tool available at http://bioinformatics.psb.ugent.be/webtools/Venn/ (Figure 5). The filtered total NEA SNPs (i.e. SNPs that passed the first four filtering steps but were not filtered based on shared polymorphism with the two high coverage genomes) were widely and densely distributed across the genome (Figure 6).

Among the SNPs found in the two high coverage dolphin genomes that had a genotype likelihood higher or equal to 0.99 for both individuals, 66% were heterozygous in one individual and homozygous in the other, 10% were heterozygous within both individuals and 24% were homozygous but with different alleles fixed within each individual. When considering the variants that were also found in the NEA dolphins, these proportions change slightly. 53% of the SNPs were heterozygous in one individual and homozygous in the other, 21% were heterozygous within both individuals and 26% were homozygous within both individuals. The mean number of individuals covered in the NEA shotgun dataset is slightly higher (7.3) for the SNPs that are heterozygous in both high coverage individuals when compared with the SNPs that are heterozygous in one individual and homozygous in the other (6.5) and homozygous in both individuals (6.4). Thus, it may be that some of these sites that are heterozygous in both high coverage individuals represent unmasked paralogues and

we caution against including these in a SNP-typing array. The list of SNPs after removal of these possible unmasked paralogues is provided in dryad.

Population genomic exploration of SNP efficacy

Using SNPs found at sites covered in at least nine individuals (corresponding to 17,866 SNPs) and assuming K=2, the inferred ancestry proportions were highly consistent with previous K=2 STRUCTURE runs on the same individuals using microsatellite genotypes (Islas 2010; Mirimin *et al.* 2011; Louis *et al.* 2014b). When including SNPs covered in less than nine individuals and for higher values of K, results became less consistent with the previous microsatellite studies. The dolphins were successfully assigned to the coastal and pelagic ecotypes (as determined previously in other studies) with high ancestry proportions apart from one individual (Figure 7). The dataset was however unable to identify the fine-scale population structure found within the coastal ecotype using microsatellite data (Louis *et al.* 2014b). Bayesian clustering analyses on microsatellite data showed that individuals sampled in the United Kingdom and Ireland formed a separate population (referred to as the "Coastal North" population) from individuals sampled in the English Channel (named the "Coastal South" population). While individuals from the coastal North population were assigned with high ancestry proportions to the coastal ecotype using the SNP dataset, all the individuals from the coastal South population showed some degree of admixture. Including SNPs with MAF < 0.1 or restricting the analysis to the SNPs only found in NEA dolphins did not improve the inference of fine-scale population structure within the coastal ecotype (data not shown). Given that the SNPs used for this inference were only covered (potentially to only 1×) in nine out of the 33 individuals, we stress that this analysis is for exploration only and some affirmation of the potential for downstream use of these SNPs. Nevertheless, given the relative concordance between the result here and previous results using microsatellite genotypes, we anticipate that SNP-typing of these markers to high coverage for all individuals would provide unprecedented resolution of population structure in this species in the NEA.

In silico cut experiments to customize and optimize RAD-seq strategies

The number of total filtered NEA SNPs recovered varies depending on the chosen RAD-seq strategy (Supplementary Table 1). As an example, the maximum number of recovered SNPs was obtained using *Sbfl* enzyme and no selection on the maximum

size of the RAD fragments. For all the strategies, a relatively low number (<3,000) of SNPs were recovered, validating our assertion that RRLs may not be appropriate for some applications for species with low genetic diversity. Nevertheless, given the fragmented nature of the bottlenose dolphin reference genome that is not yet assembled into chromosomes, the numbers of SNPs within selected fragments are likely underestimated.

Discussion

This study highlights that with careful filtering, shotgun-sequencing data can be opportunistically used for SNP discovery. Low coverage sequencing data are prone to sequencing, base-calling and mapping artifacts that might lead to false-positive polymorphism detection. The use of genotype likelihood methods that take into account the uncertainty in the data, combined with additional validation and stringent filtering steps can overcome these issues and provide high confidence in variant discovery. Several outcomes of our study support this expectation. First, there is relatively large overlap in the SNPs filtered out by each of our steps to remove variants in unmasked repeated regions, NUMTs and regions of low mappability. In addition, we found a large number of polymorphic sites (266,187 SNPs) in our dataset, which were also polymorphic in the two high coverage individuals, validating and giving high confidence to our set of SNPs. This large proportion of shared SNPs between the bottlenose dolphins from different oceans was expected given the relatively recent time to the most recent common ancestor (TMRCA) and slow molecular clocks of cetaceans (Bininda-Emonds 2007; Dornburg *et al.* 2012; McGowen *et al.* 2012; Moura *et al.* 2013). Thus, many polymorphic sites are likely to be standing genetic variation shared globally among populations, rather than derived mutations. This approach could be applied to any species for which a related species reference genome is available to map the reads.

An additional set of 174,531 SNPs that were found in the NEA dolphins were not identified as shared variants with the two high coverage individuals. Whilst false-discovery remains a possibility for these SNPs, our conservative pipeline for identifying SNPs using genotype-likelihoods combined with our stringent filtering steps will have minimized the false-discovery rate. These 174,531 SNPs may be

derived within the NEA populations and may therefore be subject to ascertainment bias if typed in populations that are not from the NEA, but are suitable for population genomic analyses specific to the NEA region. Nevertheless, some of these SNPs could be global variants that are simply by chance homozygous in the two individuals sequenced to high coverage. Ascertainment bias can arise when a small panel of individuals is used to discover SNPs or when SNPs are not geographically representative because they were discovered in one population (Morin *et al.* 2004; Nielsen 2004). In these conditions, SNPs with low minor frequency alleles are less likely to be discovered than SNPs with intermediate allele frequencies. The Site-Frequency-Spectrum (SFS) in the larger sample of individuals that is typed after the SNP discovery will then be skewed towards an excess of common alleles (Nielsen 2004; Lachance & Tishkoff 2013). Thus, inferences based on the SFS, in particular demographic history, linkage disequilibrium, diversity and F_{ST} estimates will be biased (Nielsen 2004; Clark *et al.* 2005; Albrechtsen *et al.* 2010).

For population genomics analyses on NEA bottlenose dolphins, our SNP array (440,718 total variants) has likely very low ascertainment bias given that 33 individuals from various populations (the Atlantic pelagic population and several coastal populations from the United-Kingdom, Ireland and France, see Louis *et al.* 2014a,b; Mirimin *et al.* 2011) have been used in the discovery panel. In addition, the inclusion of the rare variants discovered prior to applying the cut-off at MAF < 0.1 should allow for robust inference of most population genomic parameters in Northeast Atlantic populations. Discarding singletons and rare alleles may lead to bias when inferring demographic history based on the SFS (as they provide a signature of recent population size expansion) and population genetics parameters such as diversity and F_{ST} estimates (Nielsen 2004; Clark *et al.* 2005). Therefore, in the dryad folder, we have also provided the list of SNPs that have passed the three first filtering steps, but are not filtered based on MAF (i.e. 453,524 SNPs). We recommend that this set of SNPs is used for population genomics inferences based on the SFS or where singletons and SNPs with low MAFs are important. For population genomics studies in other areas of the distribution range of the species, we acknowledge that the global variants (266,187 SNPs) might not be optimal for some analyses that make inferences from rare variants (such as analyses that depend upon SFS-based inference). However, alternative approaches could be used such as inference of demographic history using

haplotype length (Harris & Nielsen 2013), inferences of selection on standing genetic variation (Seehausen *et al.* 2014) and test of selective sweeps based on allele frequency differentiation across populations at contiguous multiple loci (Chen *et al.* 2010). In addition, the SFS and uncertainty in the associated parameter estimates can be corrected for ascertainment bias linked to the fact that low frequency alleles have been discarded (Nielsen *et al.* 2004). The 171,573 SNPs that are ancestral polymorphisms shared between the killer whale and the bottlenose dolphin could be useful for phylogenetic studies on delphinids. Homoplasy may be an underlying process for some of these SNPs, meaning that some of these SNPs might not be broadly found in other delphinids. However, incomplete lineage sorting may also led to shared variants (as suggested for great apes, Pruefer *et al.* 2012; Mailund *et al.* 2014). A previous study (Fernández *et al.* 2016) found a high proportion of shared SNPs between white-beaked (*Lagenorhynchus albirostris*) and Atlantic white-sided dolphins (*L. acutus*) also consistent with incomplete lineage sorting.

The coverage of our data was too low and variable to infer individuals' genotypes and perform population genomic analyses that incorporated all discovered SNPs, even with methods taking uncertainty into account (Li 2011; Nielsen *et al.* 2012; Maruki & Lynch 2015). Although a clustering algorithm based on genotype likelihoods (Skotte *et al.* 2013) reliably assigned the dolphins to the coastal and pelagic ecotypes, the dataset is too incomplete to detect the finer-scale population structure within the coastal ecotype that was found using microsatellite data (Louis *et al.* 2014b). Nevertheless, this result strongly suggests that our SNP array is informative. Thus, in comparison to RRL methods such as RAD-seq and GBS, SNP discovery by shotgun sequencing (*SDBSS*) resulted only in SNP discovery rather than simultaneous SNP discovery and genotyping. Nevertheless, *SDBSS* potentially only needs to be done once for a species allowing future studies to target-sequence this reference set of SNPs using high-density SNP arrays or custom-produced baits for enrichment capture. Briefly, DNA libraries would be enriched with custom-designed biotinylated RNA baits through a hybridization reaction to capture targeted loci with sequences that are identical to the set of baits (Gnirke *et al.* 2009). Here, baits could be produced using the SNPs' coordinates on the bottlenose dolphin reference genome. For applications in species for which a reference genome is not available, the genome of a related species can be used. Enriched libraries would then be PCR-amplified and sequenced

using a next-generation technology. Currently custom baits can be used to genotype hundreds of thousands of SNPs (up to 200,000 loci) for tens to thousands of individuals. The main advantage of this approach is to target and sequence only loci that are polymorphic, which is likely to be cost effective for species with low genetic diversity such as cetaceans. For example, a recent RAD-seq study on two dolphin species by Fernández *et al.* (2016) found that 68.3% of RAD-tags were monomorphic.

This set of SNPs could be a basis for future population genomics studies on bottlenose dolphins in the NEA, and foster population structure studies that would be comparable between laboratories/geographical regions. The bottlenose dolphin is a species of strong conservation focus, as it is one of just two cetacean species listed on the European Habitats Directive. In addition, coastal populations are small, relatively isolated and have restricted home ranges which raise conservation concerns in the context of global changes (Ingram & Rogan 2002; Mirimin *et al.* 2011; Berrow *et al.* 2012; Cheney *et al.* 2014; Louis *et al.* 2014b; Louis *et al.* 2015). However, previous population genetic studies in the NEA have used microsatellites, which can constrain comparison between laboratories and datasets giving a fragmented picture of population structure. In addition, targeting-sequencing these set of SNPs using hybridization capture would also allow the investigation of local adaptation (e.g. Chen *et al.* 2010), mutation load (e.g. Peischl *et al.* 2013) and demographic history (e.g. Excoffier *et al.* 2013; Liu & Fu 2015), all of which are likely to play a role in the conservation of the recently founded and small coastal populations (Louis *et al.* 2014a,b). As stated earlier, these analyses require a large number of loci (Terhorst & Song 2015) or multiple linked variants (Chen *et al.* 2010) such as provided here.

Our shotgun approach can also be coupled with RAD-seq to optimize genotyping-by-sequencing efforts. Recently, *in silico* cut experiments have been used to estimate the expected number of SNPs under different RAD-seq strategies (DaCosta & Sorenson 2014; Lepais & Weir 2014). But this customization may not be trivial without any reference set upon which to base the optimization. We highlighted how RAD-seq strategy could be optimized by choosing the enzyme and size selection that are suitable for a given number of SNPs (Supplementary Table 1). We acknowledge that the number of SNPs recovered by the *in silico* cuts are likely underestimated due the fragmented nature of the bottlenose dolphin genome. Nonetheless, these results do provide a measure of the relative number of SNPs expected to be sequenced by

different RAD strategies, and also highlight that RAD-seq is likely to result in the discovery of orders of magnitude fewer SNPs than identified by the shotgun sequencing approach applied here. The RAD-seq customization approach could be suitable for downstream analyses for which fewer SNPs are needed, e.g. sequencing large number of individuals to investigate population structure.

Conclusions

This methodology, in particular the validation and filtering steps could be a template for SNP discovery by shotgun sequencing (*SDBSS*) based on genotype likelihoods for future studies. In contrast to RRL approaches, *SDBSS* only discovered SNPs and did not simultaneously genotype individuals. However, this approach generated a large SNP resource for NEA bottlenose dolphins that provides scope for a wide range of population genomics analyses of Northeast Atlantic bottlenose dolphins. We anticipate population-level sequencing of these SNPs will greatly elucidate the evolutionary history and provide new conservation insights into locally adaptive genomic changes in these coastal and pelagic ecotypes of bottlenose dolphins. We also highlight how this resource could be used to customize and optimize the number of SNPs targeted in different RAD-seq strategies for delphinids and other species.

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Data Accessibility

The following data will be deposited on dryad (please see the readme file for details):

- The fastq files from each NEA bottlenose dolphin
- The original NEA dolphin SNP set inferred from the genotype likelihood estimates
- The NEA dolphin SNPs after each of the filtering steps
- The total NEA dolphin filtered SNPs
- The global filtered SNPs
- The total NEA dolphin SNPs that have passed the three first filtering steps, but are not filtered based on MAF
- The global SNPs that have passed the three first filtering steps, but are not filtered based on MAF
- The total NEA dolphin filtered SNPs with the possible unmasked paralogous loci removed
- The global filtered SNPs with the possible unmasked paralogous loci removed
- The high coverage bottlenose dolphin and high coverage killer whale SNPs inferred from the genotype likelihood estimates
- The SNPs shared between either the two killer whale high coverage sequences and the filtered NEA dolphins SNPs or the two killer whale high coverage sequences and the two dolphin high coverage sequences
- The script to perform the *in silico* cuts

Author Contributions

ADF designed the study; AB, WD, RD, FG, SI, VI, ML, MN and ER collected the samples; ADF, MN, NW performed laboratory work (DNA extraction, library preparation and sequencing); ADF, SG, ML, KM, BSB and NV conducted the data treatment and analyses; ADF and ML wrote the manuscript and all authors commented on the manuscript.

Tables

Table 1. Estimates of the scale mutation rate, θ from whole genome sequences of several cetacean species using mlRho version 2.8 and nucleotide diversity (mean per-nucleotide heterozygosity) estimated in Yim *et al.* 2014.

Species	Θ	Nucleotide diversity
Bottlenose dolphins (*Tursiops truncatus*)	0.00193	0.00142
Bowhead whale (*Balaena mysticetus*)	0.0000158	NA
Finless porpoise (*Neophocaena phocaenoides)*	0.0857	0.00086
Fin whale (*Balaenoptera physalus)*	0.0569	0.00151
Killer whale – Atlantic (*Orcinus orca*)	0.000484	NA
North Pacific minke whale (*Balaenoptera acutorostrata scammoni)*	NA	0.00061

Table 2. Number of RAD loci generated and number of SNPs discovered in RAD-sequencing studies on cetaceans

Species	Enzyme	No. of loci	No. of SNPs	Study
common dolphin (*Delphinus delphis*) and harbor porpoise (*Phocoena phocoena*)	*Not1*	5,182	3,595 loci*	Viricel *et al.* 2014
killer whale (*Orcinus orca*)	*Not1*	Not reported	3,281	Moura *et al.* 2014
white-beaked (*Lagenorhynchus albirostris*) and Atlantic white-sided dolphins (*L. acutus*)	*Sbf*I-HF	179,170	52,981	Fernandez *et al.* 2016
Bottlenose dolphins (*Tursiops truncatus*)	*Sbf*I-HF	129,594	7,431	Cammen *et al.* 2015

*The number of polymorphic loci and not SNPs are reported.

Table 3. Number of SNPs that were kept and removed by each filtering step applied to the original NEA dolphin SNP set inferred from the genotype likelihood estimates

Filtering step	No. of kept SNPs	No. of removed SNPs
Mapping quality (Q<30)	503,362	27,482
Coverage (>10×)	520,512	10,332
Number of individuals (>10)	459,947	70,897
MAF < 0.1	516,494	14,350
Not variant in the two high coverage *Tursiops* genomes	334,733	196,111

Figure legends

Figure 1. Sample locations of coastal and pelagic bottlenose dolphins in the North-East Atlantic.

Figure 2. Quality of the sequenced data: a) distribution of the Phred quality scores and b) counts of sites covered by sequencing reads at different depth for each of the 33 samples. For each sequencing depth value there are 33 bars representing the 33 individuals. These graphs were plotted using the R script available at: https://github.com/mfumagalli/ngsTools/blob/master/scripts/plotQC.R.

Figure 3a. Flow chart of the SNP filtering steps.

Figure 3b. Histogram of the number of sites (i.e. bases) against the sequencing depth in the bottlenose dolphin shotgun sequencing (SGS) data.

Figure 3c. Histogram of the number of SNPs against the number of individuals in the bottlenose dolphin SGS data.

Figure 3d. Histogram of the number of SNPs against the minor allele frequency (MAF) in the bottlenose dolphin SGS data.

Figure 4. Venn diagram of the extent of overlap in the SNPs removed by the different filtering steps. Numbers indicated the amount of SNPs that were excluded by one or several filtering steps. For example, the same 11 SNPs were removed by all filtering steps and the same 388 SNPs were removed by the MAFs and number of individual filters.

Figure 5. Venn diagram of the overlap between the SNPs discovered in the NEA bottlenose dolphin shotgun sequencing data, the two high coverage bottlenose dolphin genomes and the two high coverage killer whale genomes.

Figure 6. Plot of the number of SNPs per scaffold (total variants found in the NEA bottlenose dolphin dataset) as a function of the scaffold length, up to a maximum length of 976,602 bp.

Figure 7. Ancestry proportions of individual bottlenose dolphins inferred using NGSADMIX for K=2 and SNPs found at sites covered in at least 9 individuals and comparison with the ecotype/population inferred using microsatellites in previous studies (Islas, 2010; Mirimim *et al.* 2011; Louis *et al.* 2014b). * indicated the individual that was assigned to the coastal ecotype in previous microsatellites studies

but was assigned to the pelagic ecotype in the present study. The “unknown” individual is the individual for which no microsatellite data were available.

Figures

Figure 1

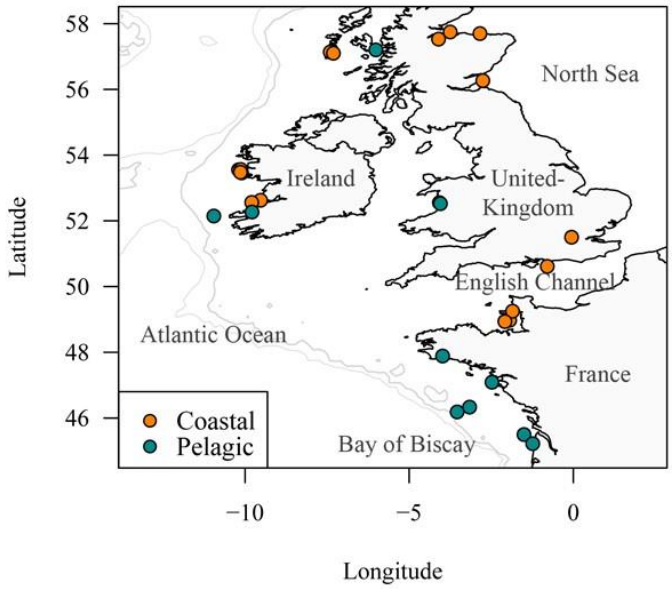


Figure 2.a

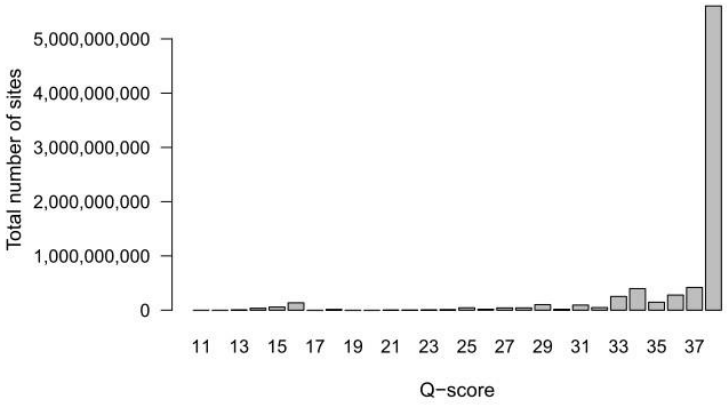


Figure 2.b

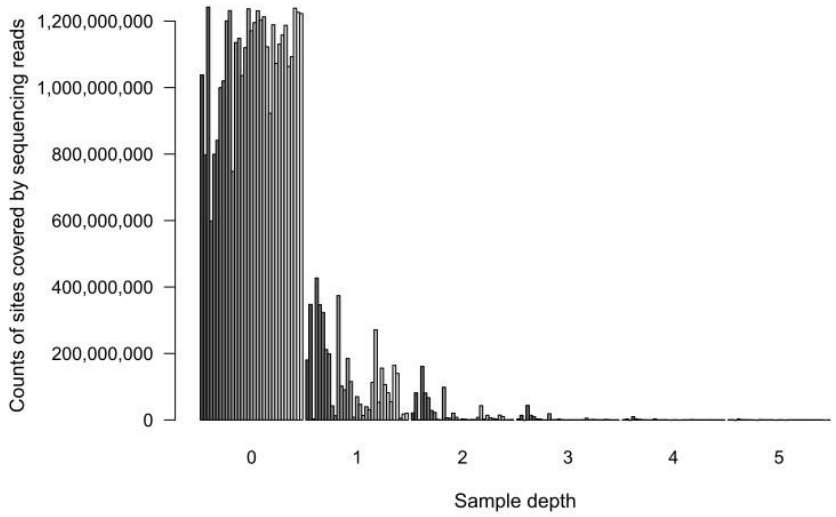


Figure 3.a

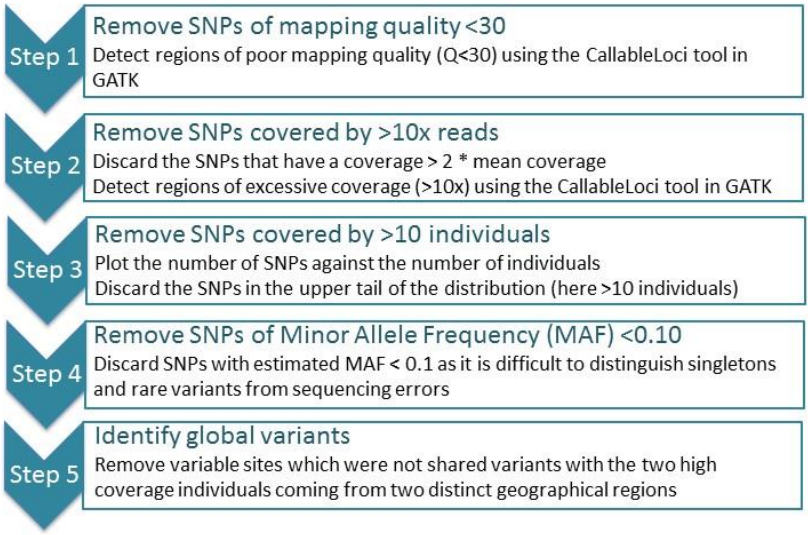


Figure 3.b

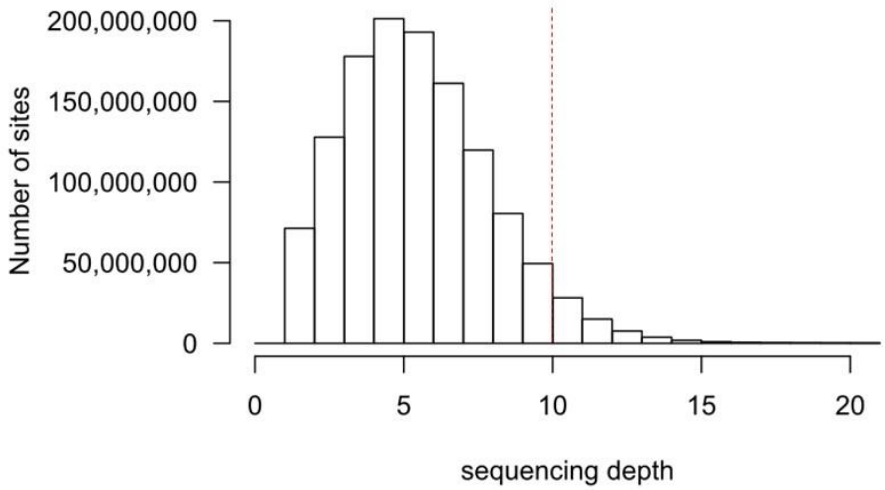


Figure 3.c

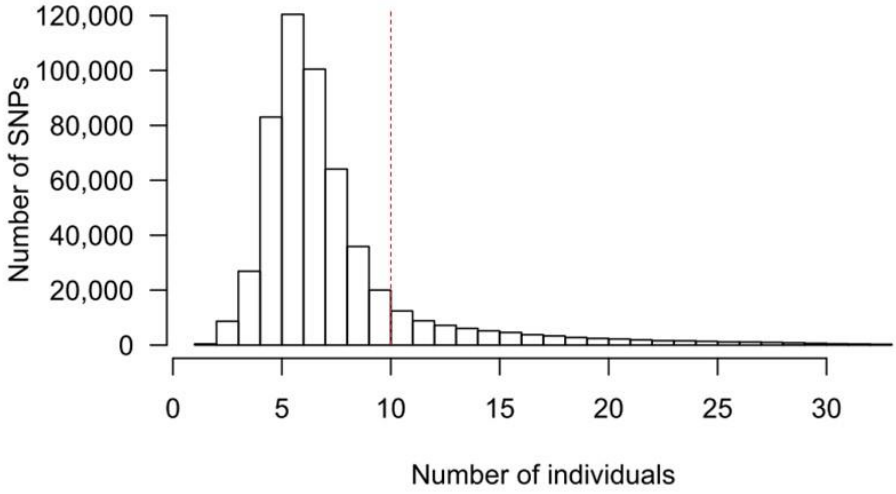


Figure 3.d

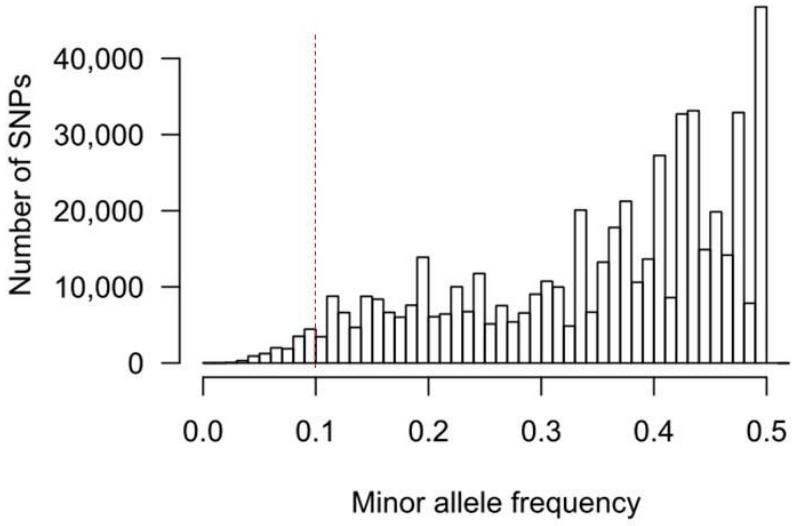


Figure 4

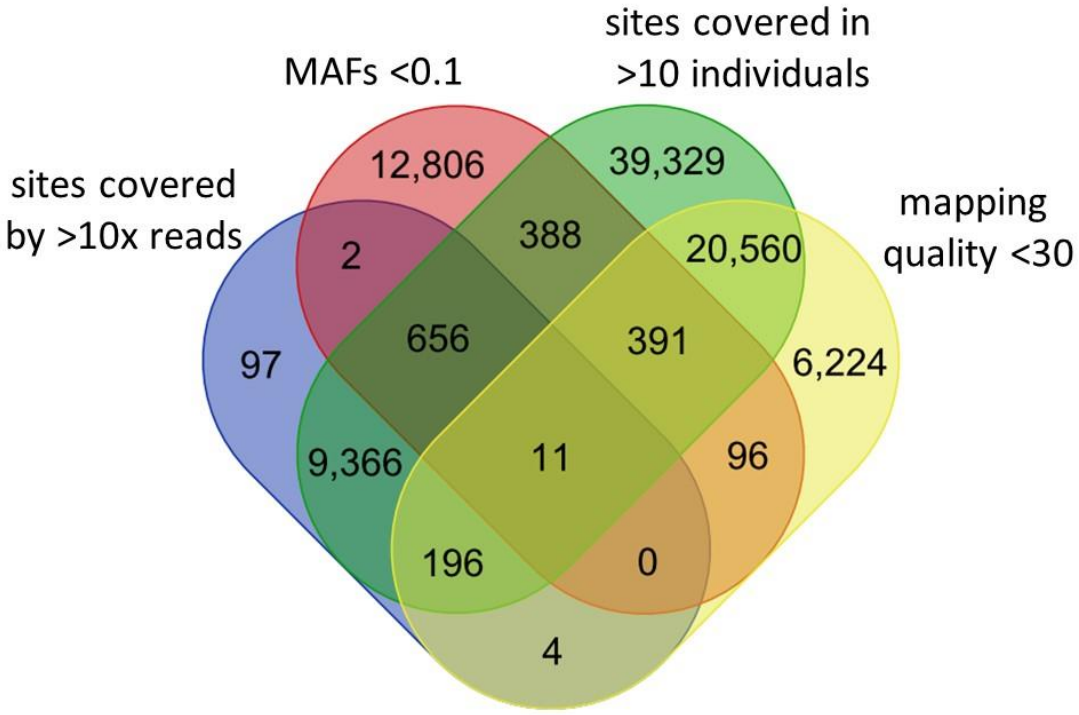


Figure 5

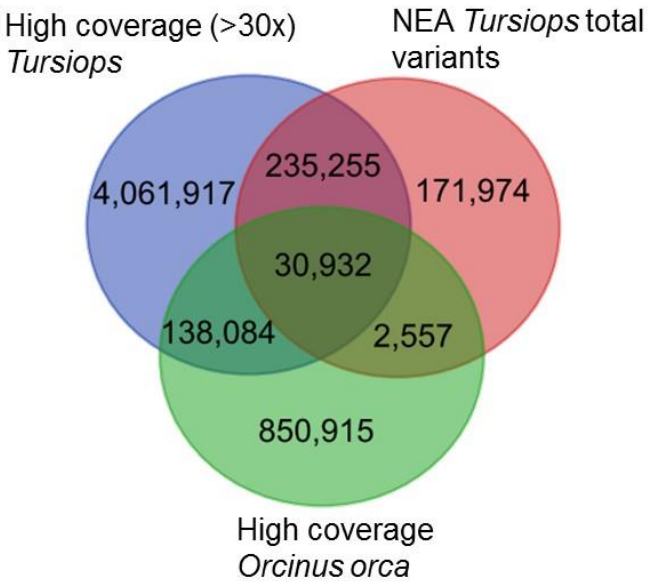


Figure 6

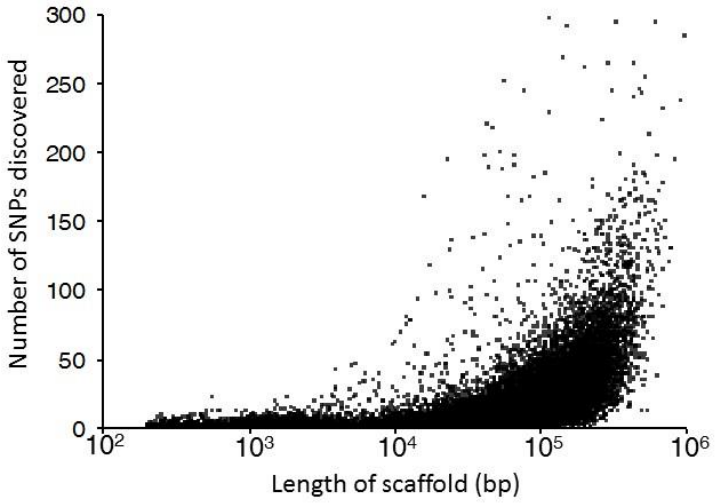
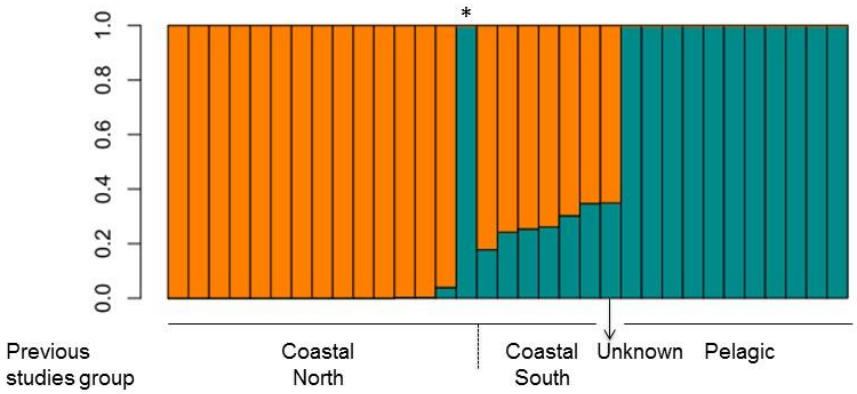


Figure 7


*
0.0
0.2
0.4
0.6
0.8
1.0
Previous studies group
Coastal North
Coastal South
Unknown
Pelagic

Supporting Information

High density, genome-wide SNP discovery in Northeast Atlantic bottlenose dolphins based on genotype likelihoods from multiplex shotgun sequencing data

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Supplementary Table 1. Results of the in-silico cut experiments to optimize RAD-seq strategies. In-silico cuts, simulating RAD-seq laboratory protocols, were generated using different enzymes and different fragment size selections. The number of our discovered filtered total NEA dolphin SNPs proximal to cut sites are reported for each RAD-seq strategy.

Enzyme	Total number of cut sites	Total number of fragments	Fragment length Min	Fragment length Max	Number of fragments passing size selection	Number of SNPs proximal to cut sites
NotI	*8084*	*3468*	0	--	3461	166
			0	1000	699	10
			0	750	638	8
			0	500	531	6
			100	--	3294	166
			100	1000	532	9
			100	750	471	7
			100	500	364	5
			100	--	3216	165
			150	1000	454	8
			150	750	393	6
			150	500	286	4
			200	--	3152	165
			200	1000	390	8
			200	750	329	6
			200	500	222	4
SbfI	*52049*	35032	0	--	35032	2831
			0	1000	3935	245
			0	750	3219	187
			0	500	2464	139
			100	--	34295	2812
			100	1000	3198	212
			100	750	2482	154
			100	500	1727	106
			100	--	34060	2806
			150	1000	2963	206
			150	750	2247	148

			150	500	1492	100
			200	--	33799	2791
			200	1000	2702	191
			200	750	1986	133
			200	500	1231	85
NotI-SbfI	*60133*	8911	0	--	8906	582
			0	1000	1120	60
			0	750	899	48
			0	500	657	38
			100	--	8718	581
			100	1000	932	57
			100	750	711	45
			100	500	469	35
			100	--	8633	577
			150	1000	847	53
			150	750	626	41
			150	500	384	31
			200	--	8569	574
			200	1000	783	50
			200	750	562	38
			200	500	320	28

Supplementary table 2. Number of sequence reads generated for each North-East Atlantic individual.

Sample ID	**Number of reads**
64	528 549
51	3 946 390
33	3 866 286
101	7 800 637
7	4 483 754
A42	210 073
A41	2 424 750
B14	2 397 060
A1	373 430
A38	4 575 655
A39	1 252 731
A4	1 479 072
SW 2001/141	1 571 916
SW1991/130	11 749 067
Tt-09-07	4 517
Tt-09-10	2 160 320
Tt-09-01	3 122 802
Tt-09-04	3 421 701
Tt-09-03	1 988 400
Tt-07-01	3 981 043
Tt-05-14	15 609 814
2007.1.179	5 196 132
2007.1.181	14 102 384
SW 2000/115	6 034 120
SW 1993/115	19 734 026
SW 1991/85	6 553 955
SW 1998/18a	1 258 237
SW 1997/171b	6 821 424
SW 2000/141e	12 826 418
SW 2004/240b	13 327 130
SW 2000/138a	15 787 409
SW 2008/93a	199 905
SW 1994/56f	25 886 537