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Phylogeography of lagenorhynchus acutus and lagenorhynchus albirostris and phylogeny of the genus lagenorhynchus

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Phylogeography of *Lagenorhynchus acutus* and *Lagenorhynchus albirostris* and Phylogeny of the genus *Lagenorhynchus*

By

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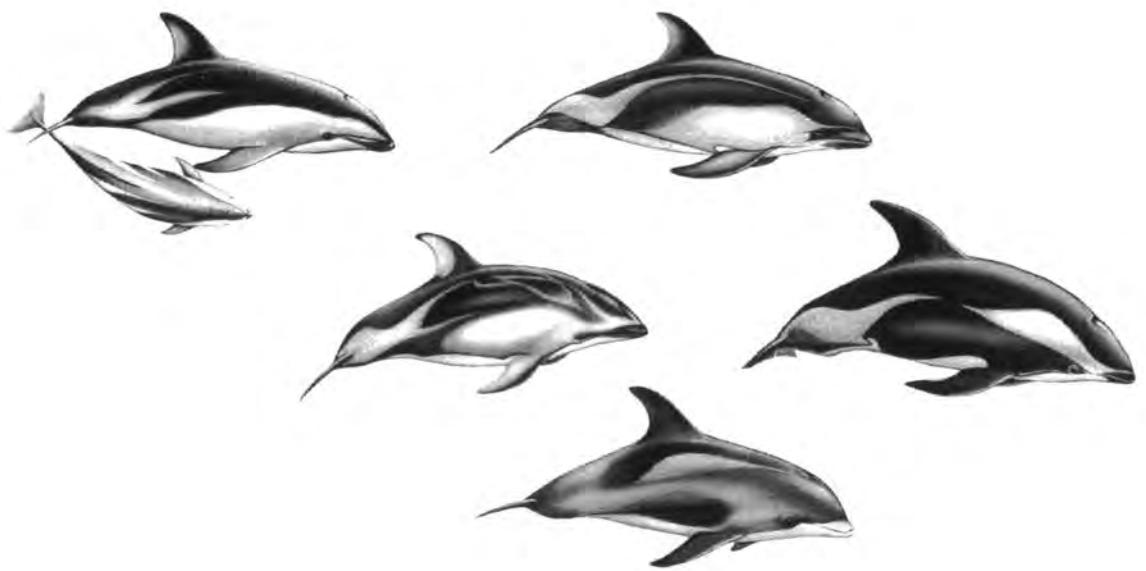
I wish to dedicate this work to all the people that have been in my life, some of them teaching me courage, others patience, others faith, others confidence; but all of them have helped me understand that there are many roads but just one way of doing things correctly—with love.

This thesis is a dedication to my father Nestor Banguera, who never had the opportunity of a higher education but who had the wisdom to teach love of knowledge to his children

To my mother María Hinestroza, an example of humility, wisdom, happiness, energy and love for life

To Trudy, my friend and advisor,
for helping and guiding me during the difficult moments in my life
with wisdom and patience
for being a true friend at every moment.

DECLARATION

The material contained in this thesis has not previously been submitted for a degree at the University of Durham or any other university. The research reported within this thesis has been conducted by the author unless otherwise indicated.

ABSTRACT

This study had two main objectives: to clarify the phylogenetic position of the species currently classified in the genus *Lagenorhynchus*, using a multilocus phylogenetic approach; and to understand the evolutionary history, population structure and phylogeography of *L. acutus* and *L. albirostris* and the processes that have influenced their distribution in the North Atlantic, using microsatellites and mitochondrial markers.

The combined phylogeny analyses performed in this study, using seven nuclear genes and two mitochondrial genes, strongly supports the artificiality of the genus and suggests deep divergences between *L. acutus* and *L. albirostris* and between these two species and the other members of the genus. Their relationships with members of the subfamily Delphininae were not corroborated, suggesting that these two species possibly deserve to be classified in a new subfamily. The total evidence phylogeny supports previous findings by other authors about the paraphyly of the other four species of the genus *Lagenorhynchus*. *L. obscurus* and *L. obliquidens* are clearly sister taxa, but they do not appear to be closely related to *L. australis* and *L. cruciger*, which are more related to *Cephalorhynchus* lineage; thus this study suggests that these species should probably be assigned to different genera.

The time of the most recent common ancestor between *L. acutus* and *L. albirostris* was placed during the late Miocene-early Pliocene (~6.53 MY ago), predating the time of splitting between these two species (~6.00 MY ago). This finding plus the placement of both species at the base of the phylogenetic tree suggest a North Atlantic origin for the ancestor of *L. acutus* and *L. albirostris*. In this study, I suggest that the ancestral populations probably migrated toward the North Pacific via the Bering Strait or via the Panamic portal during the Miocene-early Pliocene and that *L. obliquidens* probably diverged and speciated from these ancestral populations.

The evolutionary history and population structure of *L. acutus* and *L. albirostris* were assessed using a fragment of the control region of the mitochondrial DNA (d-loop) of 166 samples for *L. acutus* and 122 samples for *L. albirostris* in four geographic areas in the North Atlantic. Both species had moderate haplotypic diversity (0.9170 and 0.7320, respectively) but very low nucleotide diversity (0.0095 and 0.0056, respectively). These findings suggest that populations of both species were affected by historical bottleneck events that reduced their population sizes probably during the Plio-Pleistocene epoch and that their populations are in expansion.

When addressing the population division, the Fst values showed a clear differentiation between *L. albirostris* populations on both sides of the North Atlantic and in the eastern North Atlantic. This study also suggests the existence of one continuous population of *L. acutus* throughout its geographic range and the presence of one isolated population in the southern North Sea. These results revealed differences in the evolutionary histories of both species, which may be related to preferences in the use of habitat, and dispersion abilities. For example *L. acutus* is a pelagic species, with preferences for warm temperatures (>12°C) and deep waters, whilst *L. albirostris* is restricted to shallow and coastal areas (<120m) and prefers colder waters (<12°C).

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Many people have been involved in my academic development during my life so that it would be unfair to dedicate this thesis only to those that have been present in recent years. I know that I should name each and every one of those people; but undoubtedly their names would probably expand several pages, so I hope that they all recognize themselves in my acknowledgements.

I would like to express my gratitude to those people who with their constant support, especially during the difficult times, taught me to trust once again; to those who taught me that mistakes are part of the learning process and that not only does one have to accept them but to recognize them in order to advance along the road; to those who always trusted in me as a person and as a researcher and who encouraged me to take the decision to cross the Atlantic when my doubts held me back. To all of them, many, many thanks.

My special gratitude goes to my supervisor Dr. Rus Hoelzel for giving me the opportunity to carry out my doctorate and to learn from one of the most renowned people in the field of Molecular Ecology in cetaceans; thanks for his support, his kindness, his patience and assistance during my PhD.

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CHAPTER I: GENERAL INTRODUCTION

INTRODUCTION

The sustained anthropogenic degradation of species habitat and the subsequent loss of diversity in natural populations have increased the interest of conservationists and taxonomists in using molecular markers as a tool to evaluate the conservation status of populations and to understand the processes affecting the evolutionary history of species (Keith et al. 2005; Feral 2002; Faith 1996). For instance, microsatellite markers (bi-parental) and mitochondrial genes (matrilineal) have been used extensively in the study of cetacean populations in order to evaluate the causes of loss of diversity, to establish patterns of gene flow and to assess historical demographics. Furthermore, the analyses of the distribution of mtDNA haplotypes using coalescent theory (Kingman 1982) have allowed a better understanding of the influences that past demographic events have had on the actual distribution of species and populations (Austerlitz et al. 1997; Wakeley and Hey 1997; Roger and Harpending 1992). In addition, the field of systematics has also been enriched in the last two decades by the use of mitochondrial and nuclear genes to build phylogenies (e.g., Caballero et al. 2008; Harlin-Cognato and Honeycutt 2006; Gaines et al. 2005; Avise 2000; Pichler and Baker 2000; LeDuc et al. 1999; Hasegawa et al. 1997; Arnason and Gullberg 1996; Milinkovitch et al. 1994; Susuky et al. 1994), together with developments in statistical approaches to evaluate and interpret phylogenetic hypotheses accurately (i.e. Bayesian inferences) (e.g., Holder and Lewis 2003; Huelsenbeck et al. 2002; Yang and Rannala 1997; Rannala and Yang 1996).

In order to understand biological diversity and to provide a basis for the effective management and conservation of natural populations from an evolutionary perspective, it is essential to study the molecular ecology of populations, identify phylogeographic patterns in the distribution of lineages, and to have an accurate identification of species according to their degree of genetic variability (Price et al. 2005; Purvis et al. 2005; Dalebout et al. 2004).

This chapter presents an introduction to the three main areas of study covered by this thesis. First, the principal controversies in the classification of dolphins are addressed.

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Secondly the main theories that have been proposed to explain the antitropical distribution and radiation of several taxa are presented in order to understand the processes that have guided the phylogeography of the two unique northerly-distributed species of the genus *Lagenorhynchus* in the Atlantic Ocean (*Lagenorhynchus albirostris* and *Lagenorhynchus acutus*), and the effect that the ice ages have had on their radiation and speciation. Finally a description of the principal trends affecting the population dynamics of these species in the North Atlantic is provided, followed by a description of the main objectives to be addressed in next chapters.

1. Evolutionary relationship among species of the family Delphinidae

The evolutionary relationships among dolphins have been of great interest for decades; however, despite the efforts to provide an accurate classification of delphinids and to understand better the processes that have determined their speciation and radiation, their evolutionary history remains highly controversial and poorly understood (Price et al. 2005; LeDuc et al. 1999).

The absence of a well-supported phylogeny of Delphinidae is usually associated with the recent radiation of this group. It has been proposed that the 33 species of dolphins recognized at present were derived from a common ancestor in the mid-late Miocene around 11 million years ago (Barnes 1990). This rapid radiation and diversification gave rise to only a few diagnostic characteristics amongst dolphins, leading to difficulties in their taxonomic classification and generating controversies and disagreements in dating the splits amongst subfamilies, genera and species (Gygax 2002; Pichler et al. 2001). The main controversies in the classification of dolphins were clearly illustrated by LeDuc et al. (1999), who argued that, except for several rearrangements of the number of subfamilies and some controversies about named genera, few changes have been made in the taxonomy of dolphins in the last century. Thus the stability of this classification is not due to a true understanding of the phylogenetic relationships amongst delphinids, but to a lack of rigorous studies that would allow an accurate reclassification of the family Delphinidae using the appropriate techniques and analyses (LeDuc et al. 1999).

The classification of dolphins has been based mainly on morphological and morphometric characteristics; Mead and Brownell (1993) classified the family into 17 genera placed in six subfamilies. Three of these subfamilies have only one genus: Lissodelphininae (*Lissodelphis*) Cephalorhynchinae (*Cephalorhynchus*) and Orcaellinae (*Orcaela*). The other three subfamilies have 3 genera or more: Stenoninae (*Steno, Souza* and *Sotalia*), Delphininae (*Lagenorhynchus, Gramphus, Stenella, Tursiops, Lagenodelphis* and *Delphinus*) and Globicephalinae (*Peponocephala, Pseudorca, Feresa, Globicephala and orca*).

The first molecular study to propose a significant modification in the above classification was presented by LeDuc et al. (1999). Based on their studies with the full mitochondrial Cytochrome b gene (Cytb), these authors recommended examining several evolutionary relationships in the family Delphinidae. They proposed that the genera *Lagenorhynchus, Cephalorynchus and Lyssodelphis* be placed in a new subfamily Lissodelphininae, which would integrate 10 antitropically distributed species: *Lissodelphis borealis, L. peronii, Cephalorhynchus commersoni, C. eutropia, C. hectori, C. heavisidii* and four species of the genus *Lagenorhynchus* (*L. obliquidens, L. obscurus, L. cruciger and L. australis*). The two North Atlantic species of the genus *Lagenorhynchus* (*L. acutus* and *L. albirostris*) were excluded from the subfamily as no close relationships were found between these two species and the other members (see introduction to next chapter). Although the species placed in the new subfamily Lissodelphininae are all morphologically distinguishable, a clear picture of their relationship is still lacking (Pichler et al. 2001).

Another group of importance for reclassifying the family Delphinidae is the group composed of the genera *Delphinus, Tursiops* and *Stenella*. The species in these genera have been subjected to reclassification several times; for example sympatric species in the genus *Delphinus* are distinguishable for certain morphological characteristics (e.g., colour patterns, external morphology and cranial characteristics) but not for others (e.g., rostral length) (Kingston and Rosel 2004). Some species of the genus *Delphinus* and *Tursiops*, considered morphotypes of the same species, have been recently claimed to be separate species based on molecular markers (Rosel et al. 1994; Natoli et al. 2004).

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Since the reclassification of Delphinidae proposed by LeDuc et al. (1999), a few new molecular studies have been carried out to understand the phylogenetic relationships amongst dolphins (e.g., Caballero et al. 2008; May-Collado and Agnarsson 2006; Harlin-Cognato and Honeycutt 2006; Pichler et al. 2001). Except for the studies by Caballero et al. (2008) and Harlin-Cognato and Honeycutt (2006), most of them have been based on a single mitochondrial gene and have failed to resolve the most conflictive clades within the family. Therefore there is still no agreement about the phylogenetic relationships among all members of the family Delphinidae (Kingston and Rosel 2004).

In this study a combined phylogenetic analysis of seven nuclear genes and two mitochondrial genes was used to contribute to the resolution of the phylogenetic relationships among the six species of the genus *Lagenorhynchus*, which provided insights into their relative position within the wider Delphinidae phylogeny.

2. Phylogeography: Understanding the relationships between the demographic history of populations and distribution of gene lineages within species

The links amongst molecular data, biogeographical patterns and historical events have facilitated the study of evolutionary processes (e.g., Emerson and Hewitt 2005; Knowles and Richards 2005; Avise 2000; Avise 1994). Statistical approaches and mathematical models allow the signature of historical and demographic processes to be determined from the analysis of DNA (Avise 2000; Avise 1994). For example, hypotheses about the genetic consequences of ice ages (i.e. the isolation of populations in refugia during interglacial periods) can be tested using the distribution of pairwise differences amongst DNA sequences (mismatch distribution). This analysis will show a unimodal distribution in cases of strong bottlenecks followed by population expansion and a bimodal distribution in cases where populations have remained stable during long evolutionary periods (Schneider and Excoffier 1999).

On the other hand, the application of the coalescence theory to sequence data makes it possible to trace the shared history of genes back into the past, to the time that they coalesced and reached a common ancestor (Emerson et al. 2001). This theory predicts that

the growth or decline in a population and the temporal scales in which they take place (i.e. ancient or recent) is recognizable in the pattern of DNA substitution amongst sequences drawn from a population (Emerson et al. 2001). Thus, it is possible to estimate population parameters within species or amongst closely related species (e.g., migration rates, recombination, effective population sizes, etc.) from gene sequences and to build a gene genealogy or tree. The shape of the tree will depend on the demographic history of the populations under study (Posada and Crandall 2001; Austerlitz et al. 1997; Wakeley and Hey 1997).

Phylogeographic studies and coalescent approaches address a variety of population genetics and evolutionary hypotheses, not only within species but also amongst different, similarly distributed taxa (comparative phylogeography) (Arbogast and Kenagy, 2001; Posada and Crandall 2001, Avise 2000). Although the phylogeographic patterns in marine species can be difficult to interpret, especially due to the absence of barriers to gene flow in the marine environment (Pastene et al. 2007), these studies have recently increased in number, in particular addressing hypotheses related to the consequences of climate changes (e.g., interglacial periods and changes in water temperatures) on the pattern of genetic variation in marine species (e.g., Harlin-Cognato et al. 2007; Pastene et al. 2007; Cassens et al. 2005; Hayano et al. 2004; Harlin et al. 2003; Hewitt 2004, 2000; Pichler et al. 2001; Avise et al. 1998).

The interpretation of phylogeographic patterns within the context of evolutionary and biogeography models is important, not only for recognizing the relationships between population structure and demographic structure and understanding the effect of past events in the actual distribution of species, but also for identifying accurate scales of management and conservation in natural populations (Arbogast and Kenagy 2001; Avise 2000; Avise 1994; Hoelzel 1998). For example, Pichler et al. (2001), who combined data on the demographic history and historical processes with the analysis of the mtDNA control region, hypothesized that the radiation of the genus *Cephalorhynchus* into the Southern Hemisphere was possibly constrained by the sub-Antarctic current system, which may have acted as a barrier to more northerly dispersal. These authors also suggest that the

split of *Cephalorhynchus* into two South American species (*Cephalorhynchus eutropia* and *Cephalorhynchus commersoni*) was the result of glaciations in Tierra del Fuego isolating a population in the northern part of their range. A similar pattern of dispersion and speciation has been also suggested for the species of the genus *Lagenorhynchus* distributed in the southern hemisphere (Harlin-Cognato et al. 2007; Cassens et al. 2003).

In another phylogeographic study, Pastene et al. (2007) gave new insights into understanding the mechanism of speciation in populations of minke whales (*B. acutorostrata*) and the timing of the split between *B. acutorostrata* and *B. bonaerensis*. Based on mtDNA control region sequence data, the authors suggest divergence in the Southern Hemisphere during the Pliocene, an epoch of global warming; contrary to previous suggestions that the subdivision was a consequence of tropical barriers between northern and southern populations (see Pastene et al. 2007). The speciation of *B. acutorostrata* was probably followed by a rapid radiation of the extant populations toward the North Atlantic and North Pacific after the end of the Pliocene global warming period. The authors also suggest that the population subdivision between the two oceans was helped by pronounced cooling during the Late Pliocene/Early Pleistocene, and the differences between northern and southern populations were mainly maintained by periods of glacial cycles and intervening warm periods.

The pattern of antitropical distribution (referring to related taxa distributed on opposite sides of the equatorial belt) has been one of the most intriguing in the distribution of marine mammals (Cipriano 1997; Lindberg 1991). At least three odontocete families (Ziphiidae, Phocoenidae and Delphinidae) have members distributed in opposite hemispheres (see Cipriano 1997; Davies 1963). Several hypotheses have been proposed regarding the causes of this pattern of distribution on marine taxa. Davies (1963), for example, proposed that it was a consequence of glacial cycles during the Plio-Pleistocene epoch and intervening warming periods in the ocean during the mid-Miocene epoch, which isolated previously panmictic populations into different hemispheres (see Cipriano 1997). However, White (1986) suggested that the antitropical distribution was the consequence of the global depression in temperatures during the early Oligocene epoch, which allowed the spread of temperate-adapted organisms into low latitudes. These low

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temperatures probably persisted until the mid-Miocene. Thus antitropical distributions became established as rising tropical temperatures displaced taxa into higher latitudes.

To date the phylogeographic studies in the genus *Lagenorhynchus* have been focused mainly in two species: *L. obscurus* and *L. obliquidens* (Harlin-Cognato et al. 2007; Hayano et al 2004; Cassens et al. 2003; Hare el al. 2002); with the exception of the preliminary study by Cipriano (1997) which included four species of the genus *Lagenorhynchus*, no studies have addressed hypotheses about the antitropical distribution of all six species of the genus from a phylogeographic perspective. Furthermore, no studies have addressed hypotheses about the radiation of these six species and their possible routes of migration and dispersion. Nor have studies been carried out on the phylogeography of the two North Atlantic species of the genus (*L. acutus and L. albirostris*). These data are key to a fuller understanding of their pattern of antitropical distribution and the effect of climatic changes on processes leading to their radiation and distribution. If the differentiation of the two northerly distributed species of the genus from the other species is confirmed in this study (as suggested by LeDuc et al. 1999 and Harlin-Cognato and Honeycutt 2006), new phylogeographic hypotheses could be tested about the effect of climatic changes on origin, radiation and speciation in delphinids and about the ancestral relationships between and amongst closely related species of the currently recognized genus *Lagenorhynchus*.

A further objective of this study will be to interpret the demographic history of *Lagenorhynchus acutus* and *Lagenorhynchus albirostris* from phylogenetic data (intraspecific phylogeography) in the context of possible range and population dynamics since the last ice age, and to test hypotheses about colonization routes, migration processes and population division in their distribution range. A comparative study of the phylogeography of each species (comparative phylogeography) will provide an understanding of the evolutionary forces that have shaped the distribution and population structure of each of these two species in the North Atlantic. Similar population structure patterns will indicate similar processes in their evolution, whereas a dissimilar pattern in

their current distribution and population structure could indicate different responses of these species to the evolutionary forces shaping their demographic history.

3. Population genetics: Studying genetic diversity within and amongst populations as a tool for future conservation plans

Anthropogenic disturbances have been recognized as the primary cause of decline in wild animal populations worldwide (e.g., Lacy and Martin 2003; Tallmon et al. 2002). The main concern in the conservation of cetacean populations has been the effect that pollution, habitat destruction, competition for prey resources and direct takes has had on the viability of species. Bycatch (unintended capture while fishing) is now recognized as being one of the key threats to cetacean species, and as being the major cause of human-induced mortality amongst aquatic organisms. It is well known that the high levels of induced mortality, especially in coastal species of dolphins, have threatened many populations that could represent different evolutionary units or incipient species (Cassens et al. 2005; Rosel et al. 1999; Castello 1996; Mitchell and Reeves 1981). In addition, population genetic studies of cetaceans have shown that the reduction in population sizes (bottleneck) in species affected by commercial hunting or accidental takes can have serious affects on the long-term viability of the population, and has the potential to influence the density, behavior, demographics and long-term survival of species (Pichler and Baker 2000; Rosel et al. 1999). This is especially noticeable in cetacean populations because their longevity and relatively low reproductive rates make them more vulnerable to the increasing influences of humans (Castello 1996).

Well-known examples of high mortality rates in fishing nets impacting genetic variability have been reported for porpoises [*Phocoena phocoena* (Rosel et al. 1999) and *Phocoena sinus* (Rojas-Bracho and Taylor 1999; Rosel and Rojas-Bracho 1999)]. *P. phocoena* forage in one of the most perturbed habitats in the world, and it has been estimated that fisheries throughout the Northern Hemisphere have accidentally killed thousands of them in recent decades. This has led to severe population declines (Elliot et al. 2003) and *P. sinus* is considered to be in risk of extinction in its distribution range as a result of gillnet bycatch (Rojas-Bracho and Taylor 1999). A large reduction in effective population sizes

not only produces a reduction in genetic diversity (increasing the rate of genetic drift), but also increases inbreeding (Brakefield and Saccheri 1994), increasing the risk of extinction (e.g., Garza and Williamson 2001; Amos and Balmford 2001, Luikart and Cornuet 1998; Amos and Harwood 1998). Consequently, the identification of loss of genetic variability in populations and the identification of management units have become important goals for conservation biologists.

Two types of molecular markers have been used extensively in these studies: the mtDNA control region and microsatellite DNA markers. The latter is currently the most widely used genetic marker in molecular ecology, especially because of its ubiquity, high level of polymorphism and biparental heredity. The wide use of microsatellites is also supported by the increasing development of population genetic approaches associated with them (e.g.,Van Oosterhout et al. 2004). Microsatellites also allow recognition of recent population declines, often associated with strong human pressure on habitats and populations (Luikart and Cornuet 1998; Cornuet and Luikart 1996).

Another objective in this study will be to evaluate the processes that have affected genetic variability in populations of *L. acutus* and *L. albirostris* at the nuclear (using microsatellite DNA loci) and mitochondrial levels to determine whether or not the population sizes of these species have declined recently due to bottleneck events.

In summary, the thesis will address three main objectives:

1. To test the hypothesis, supported by some earlier works, that the genus *Lagenorhynchus* is polyphyletic, using a combined data set of nuclear and mitochondrial genes and assessing their position within the family Delphinidae. An interpretation of the evolutionary relationships amongst the six species of the genus, based on their pattern of distribution and the changes in the marine environment during the last geological eras (i.e., Miocene-Pliocene-Pleistocene), will allow a better understanding of the patterns of dispersion and speciation of these species.

2. To assess the hypothesis of post-glacial expansion *in L. acutus* and *L. albirostris* in the North Atlantic as reported for other marine species in this region. Nuclear and mitochondrial genes will be used to address their demographic history from phylogenetic data for both species (intraspecific phylogeography) within the context of possible range and population dynamics since the last ice age.

3. To identify the processes involved in generating the pattern of genetic diversity found in these species, and test the hypothesis that habitat preferences leads to different patterns of population structure as suggested in earlier studies on different delphinids species. The identification of the degree of genetic variability and the processes affecting this variability will be important towards the development of effective conservation and management strategies for *Lagenorhynchus acutus* and *L. albirostris* in the North Atlantic and North Sea.

CHAPTER II: PHYLOGENETIC RELATIONSHIPS AMONGST SPECIES OF THE GENUS *LAGENORHYNCHUS* BASED ON MITOCHONDRIAL AND NUCLEAR GENES

INTRODUCTION

Lagenorhynchus represents the most numerous genus of the Delphinidae family, commonly classified into the subfamily Delphininae, with six species (Fraser 1966). *L. albirostris* (Gray 1846) and *L. acutus* (Gray 1828) are distributed in the polar and sub-polar waters of the North Atlantic and North Sea. *L. obliquidens* (Gill 1865) is confined to the North Pacific in the Kamchatka Peninsula, Russia, Alaska, Japan (North to South) and south of Baja California. *L. cruciger* (Quoy and Gairmard 1824) has a circumpolar distribution, being found along the coasts of the Antarctic and Sub-Antarctic. *L. australis* is restricted to the cold waters of southern South America (South of Chile and Argentina, around Tierra del Fuego, Beagle Channel and the Falkland Islands); while *L. obscurus* (Gray 1828) is distributed around South America, south of Africa and coastal regions to the south of Australia, Tasmania, New Zealand and the Kerguelen Islands in the South Indian Ocean (Leatherwood et al. 1991; Gaskin 1992) (Figure II.1).

These species were placed together because of similarities in colouration, skull and beak shape (Mitchell 1970; Fraser 1966); however Miyazaki and Shikano (1997) found that *L. acutus* and *L. albirostris* have more shared morphometric characteristics between each other than with other members of the genus. LeDuc et al. (1999), using the complete Cytochrome b gene (Cytb), showed evidence for the possible artificiality of the genus as previously suggested by Cipriano (1997) in his preliminary study on the phylogeography of the genus.

In their study LeDuc et al. (1999) proposed excluding the six species of the genus from the subfamily Delphininae and placing four of them (*L. obliquidens, L. obscurus, L. cruciger and L. australis*) into the subfamily Lissodelphininae, which previously contained only the genus *Lissodelphis*, together with the genus *Cephalorhynchus*. The authors also stated that the two remaining species (*L. acutus* and *L. albirostris*) are not closely related to each other or to any of the other *Lagenorhynchus* species. They suggested that *L. acutus* be assigned to the genus *Leucopleurus* (*Leucopleurus acutus*), that *L. albirostris* retain the name of the genus as the type species and that the four

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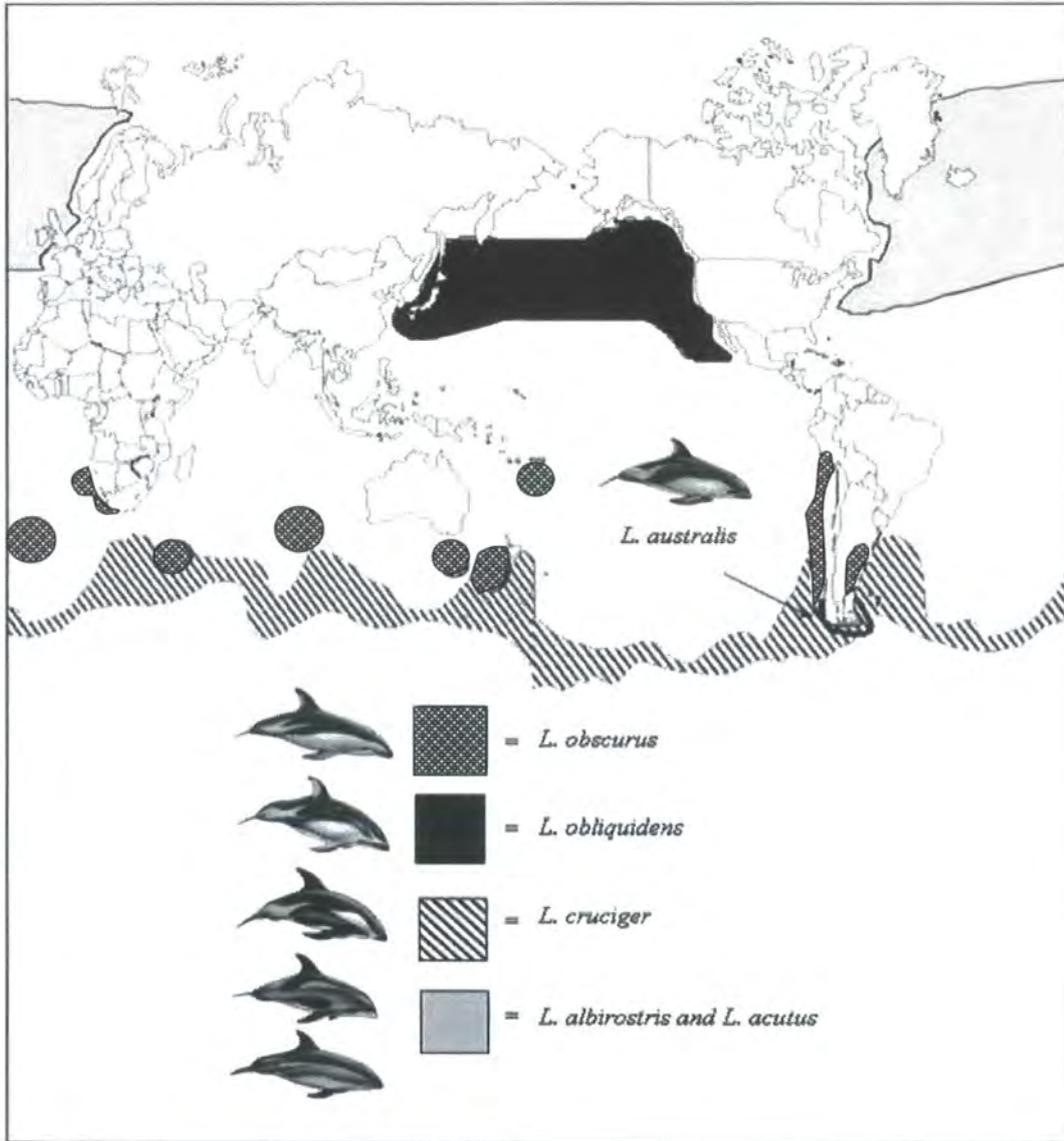
Figure II.1. Distribution of the genus *Lagenorhynchus.* Figure adapted from Harlin (2004).

remaining species (*L. obliquidens*, *L. australis*, *L. obscurus* and *L. cruciger*) be grouped into a new genus, *Sagmatias* (Cope 1886).

The evolutionary pattern found by LeDuc et al. (1999) was partially corroborated by Pichler et al. (2001) when using the control region of the mtDNA; by May-Collado and Agnarsson (2006) using Bayesian analysis of partial Cytb sequences; and by Harlin-Cognato and Honeycutt (2006) using a multigenic phylogeny combining the Cytb gene, the mitochondrial control region and two nuclear genes (Actine and RAG protein).

Although the study by LeDuc et al. (1999) gave a new insight into the further development and understanding of the phylogenetic relationships amongst dolphins, it fell short in two respects. First, the majority of intrageneric relationships were not resolved in their phylogenetic analysis, and most of the relationships amongst *Lagenorhynchus* species were not determined (Figure II.2a). Secondly, the authors proposed the revision of the family based on a single mitochondrial gene (Cytb). The phylogenies presented by Pichler et al. (2001) and May-Collado and Agnarsson (2006) had similar limitations as LeDuc et al.'s (1999) work. In the phylogenetic analysis carried out by Harlin-Cognato and Honeycutt (2006), the combination of nuclear genes did not fully resolve the relationships amongst *Lagenorhynchus* spp., and the D-loop region was a source of conflict in the combined phylogeny as discussed by the authors (see Figures II.2b and II.2c).

It is well known that single mtDNA phylogenies have failed to provide reliable, well-supported trees in phylogenetic analyses (Price et al. 2005; Farias et al. 2001). Phylogenies derived from a single mitochondrial gene can be inaccurate due to heterogeneity in mtDNA substitution rates (Ballard and Rand 2005; Grassly and Holmes 1997; Galtier and Gouy 1995) or selection at that locus or linked loci in the mitochondrial genome (Dean and Ballard 2004; Goldstein and Harvey 1999). Moreover, mtDNA becomes monophyletic faster than nuclear genes because its effective population size
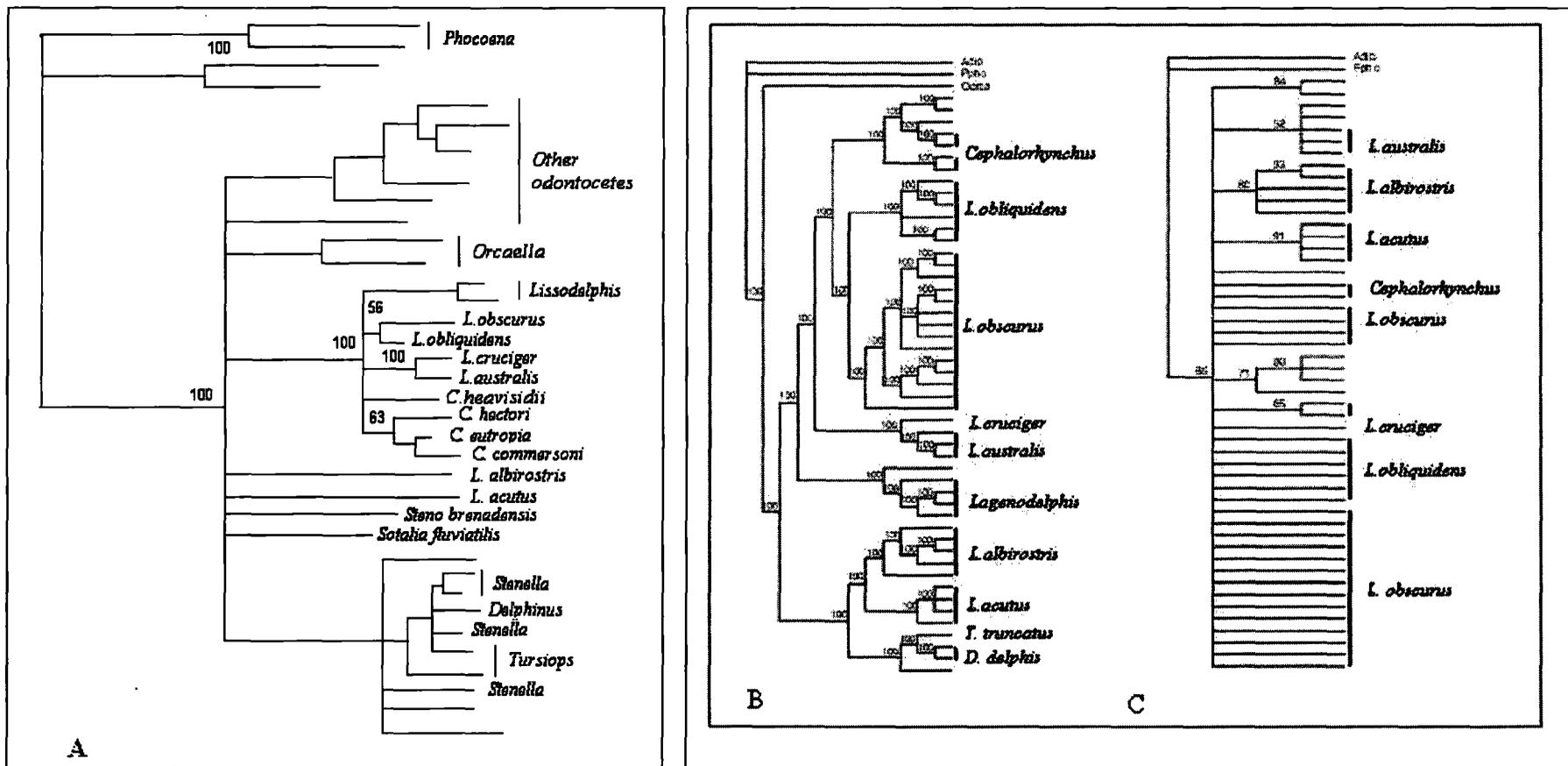
Figure II.2. Phylogenetic relationships amongst species of the genus *Lagenorhynchus*. Figure **A.** shows the relationships found by LeDuc et al. (1999) using the Cytb gene; Figures B and C show the phylogenetic relationships found by Harlin (2004) using the control region and Cytb gene (**B**) and using two nuclear genes (ACT and RAG) in **C.**

is one-fourth that of nuclear genes (see review in Moritz et al. 1987). Thus inferences of monophyletic groups at the species level based on mitochondrial genes can be erroneous (Hudson and Coyne 2002; Ballard and Rand 2005).

In recent years a number of studies have also shown that both mitochondrial and nuclear genes can lead to markedly different phylogenies as a consequence of different inheritance pathways, selection pressures and differences in responses to evolutionary processes in both genomes (Philippe and Telford 2006; Ballard and Rand 2005; Zhang and Hewitt 2003; Shaw 2002).

Multilocus or total-evidence phylogenies have provided new insights into the phylogeny of many organisms in recent years. It is generally accepted that several nuclear loci can provide independent estimates of phylogenetic relationships to corroborate or refute previous mtDNA phylogenies and thereby avoid the effects of selecting at a particular locus (e.g., Caballero et al. 2008; Gaines et al. 2005; Reyes et al. 2004; Weins 1998; DeSalle and Brower 1997).

The use of multigenic phylogenies to address the current controversies in the evolutionary relationship amongst species of the genus *Lagenorhynchus* and between closely related species will give a new insight into the phylogenetics of dolphins and will allow a better understanding of the radiation and diversification of this genus. Given the foregoing, the main aim of this chapter is to address the evolutionary relationships amongst the six species of the genus *Lagenorhynchus* and their relative position within the delphinids phylogeny. In this study a multigenic approach will be used to test two hypotheses, supported by earlier works: the polyphyly of the genus *Lagenorhynchus* and the paraphyly of the proposed new genus (*Sagmatias*) (LeDuc et al. 1999).

MATERIALS AND METHODS

1. Samples and DNA extraction

In order to study the utility of nuclear markers in resolving phylogenetic relationships amongst species of the genus *Lagenorhynchus*, DNA samples for each species of this genus, one species of the genus *Cephalorhynchus* (*C. commersoni*), and three species of the subfamily Delphininae (*Stenella coeruleoalba*, *Tursiops truncatus* and *Delphinus delphis*) were included in this study. The species *P. phocoena*, considered the sister group of Delphinidae, was included as an out-group.

DNA and tissue samples were obtained from different sources: DNA samples of *T. truncatus*, *D. delphis*, *L. acutus and L. albirostris* belong to the DNA archive of the Molecular Ecology group at the University of Durham; tissue samples of *L. cruciger, L. australis and C. commersoni* were provided by Dr. Enrique Crespo from the Centro Nacional Patagonico (CONICET) in Argentina. Sequences of *L. obliquidens* for all genes analyzed in this study that were not already published in the GenBank database were obtained in collaboration with Dr. Azusa Hayano from Kyoto University, and tissue samples from *S. coeruleoalba* were provided by George Gkafas from the University of Thessaly (Greece).

2. DNA extraction and genes amplification

Total genomic DNA was extracted from tissue following the procedure recommended by Hoelzel and Green (1998). Tissues samples were cut into small pieces and placed in an autoclaved Eppendorf® containing 250-500 μl of digestion buffer (50mM tris pH 7.5, 1mM EDTA, 100 mM NaCl, 1% SDS and 25 μl of Proteinase K). Samples were digested over night at 55°C, and DNA was isolated using a standard phenol: chloroform protocol. DNA was quantified in 1.0% agarose gel stained with ethidium bromide and stored at 20°C until use.

After DNA extraction, different primers previously published in the literature (Table II.1) were used to amplify 5 nuclear genes and 1 mitochondrial gene using Polymerase Chain Reaction (PCR, Saiki et al. 1988): (i) A section of Exon 1 of the inter-photoreceptor retinoid binding protein gene (IRBP), which is a single-copy gene that functions in the regeneration of rhodopsin in the visual cycle (Stanhope et al. 1992; Springer et al. 1997). (ii) Exon 28 of the gene encoding the von Willebrand Factor (vWF), which is the longest exon of a single functional nuclear copy gene for a plasma glycoprotein that functions in blood clotting (Porter el al. 1996). (iii) The α lactalbumin gene (LAC), that codes for a secretory protein of the mammary tissue (Waddell et al. 2000, see details in Milinkovitch et al. 1998) (iv) The Ca+ calmodulin-dependent kinase (CAMK) gene, which is a single-copy gene for a tissue-restricted protein kinase (Lyons et al. 1997; Bland et al. 1994) (v) The intron 6 of the beta-hexosaminidase beta chain gene (HEXB), which is a specific precursor glycoprotein involved in the hydrolysis of β-linked N-acetylhexosamine (Lyons et al. 1997), and (vi) The 16s rRNA gene.

After standardizing optimal annealing temperatures and magnesium concentration, genes IRBP, 16s rRNA, LAC and vWF were amplified under the following conditions: 94° 2 min followed by 32 cycles of 94°C 15sec, specific annealing temperature for 15 sec. and a final extension of 72°C 30 sec. CAMK and HEXB genes were amplified using 95°C 2 min, followed by 35 cycles of 95°C 30 sec, 30 sec at specific annealing temperature, 72°C 2 min, a final 5 min extension at 72°C. Annealing temperatures for each gene are indicated in Table II.1.

PCR products were sequenced in both directions on an ABI 377 automated sequencer; internal primers were used to sequence long amplicons in CAMK and HEXB (CAMK: i. 5' AAAAGTTGCTACCATTAT 3', ii. 5' GATCAGCAGCACCTCCAT 3', iii. 5' CTTTCTCAAAAGTCCTGCA 3', iv. CTTTCTCAAAAGTCCTGCA; HEXB: genes (i. 5' TATATAGCTGCCCATCCC3' ii. 5' CAGGAGACAGAAGAACAT 3' iii. 5' CTCCAAGGCTTTGATGTAGT 3'). Sequences were aligned using the Clustal X programme v. 1.83 (Thompson et al. 1997) and edited and compiled using the programme

Table II.1. Nuclear and mitochondrial genes obtained in this study.

GENE	Number of nucleotides	References	Annealing T°C	Additional sequences from GenBank data base (accession numbers)
LAC	1058	Milinkovitch et al. 1998	54	AF304089, AF228410, AJ007811
CAMK*	2113	Lyons et al. 1997	62	AF140819, AF140813
vWF	1191	Porter et al. 1996	64	AF061060
IRBP	1103	Stanhope et al. 1992	64	AF304077, AF304078
HEXB**	2018	Lyons et al. 1997	61	AF140841
16s rRNA	509	Palumbi et al. 1991	52	DDU 13106, LOU13114, Z18649, PPU13121, AJ554061, LAU13113, AY770538, AJ010816

*Sequences spans one or more introns (see Gaines et al. 2005).
**Sequence includes a SINE insertion of 107 bp.

Chromas Pro (www.technelysium.co.au). All sequences were subjected to a Blast search to verify sequence orthology.

A total of 7993 bp were successfully obtained from 6 genes and two individuals of each species involved in this study: 1103 bp of the IRBP gene, 2113 bp of CAMK gene, 1191 bp of the vWF gene, 1058 bp from LAC gene, 2019 bp of the HEXB gene and 509 bp segment of the 16s rRNA gene. In addition 2427 bp were obtained from the GenBank data base: 1140 bp of the Cytochrome b gene (Cytb), 474 bp of the Recombination Activator Protein (2) gene (RAG2) and 813 bp of one intron from the nuclear muscle Actin gene (ACT) (See Table II.2). Thus, a total of 9 genes and 10420 bp were included in this study. With the exception of two sequences from the 16s rRNA gene that were substantially different in *L. obliquidens*, all other individuals from the same species had similar or identical sequences; therefore only one sequence per species was included in all analyses.

3. Phylogenetic analysis

3.1. Description of phylogenetic methods

Different approaches have been traditionally used to build phylogenetic trees and to infer phylogenies of related groups. The commonest methods are algorithm methods such as Neighbor joining algorithm (NJ) and tree searching strategies based on optimality criteria such as Maximum Parsimony (MP) and Maximum Likelihood (ML) (Holder and Lewis 2003; Swofford et al. 1996). More recently, the use of the Bayesian statistic in phylogenetic analysis has become popular. Although its use in phylogenetics is highly controversial (see review in Alfaro and Holder 2006 and Huelsenbeck et al. 2002), the use of this methodology has been considered the primary methodological innovation in phylogenetics (Huelsenbeck et al. 2002).

- **Neighbor Joining method (NJ)**

The NJ analysis (Saitou and Nei 1987), which is the fastest of all methods, is mainly recommended when the divergence between sequences is low. This method converts DNA sequences into a distance matrix, which represents an estimate of the evolutionary distance

Table II.2. Accession numbers for samples extracted from the NCBI GenBank data base.

Species	Genes		
	Cytb	**RAG**	**ACT**
Delphinus delphis	EF093031	EF093079	EF092991
Lagenorhynchus obscurus	EF093055	EF093104	EF093008
Phocoena phocoena	EF093010	EF093057	EF092971
Lagenorhynchus albirostris	EF093018	EF093064	EF092979.
Tursiops truncatus	EF093029	AY011968	EF092989
Stenella coeruleoalba	AF084081		
Lagenorhynchus obliquidens	EF093041	EF093091	AF140831
Lagenorhynchus acutus	EF093022	EF093069	EF091836
Lagenorhynchus australis	EF093035	EF093085	EF092997
Cephalorhynchus commersoni	AF084073	EF093070	EF092984
Lagenorhynchus cruciger	AF084068		

between sequences (Holder and Lewis 2003). However, the method may fail to rebuild accurate phylogenies, primarily because the observed differences between sequences do not always reflect the evolutionary distances between them and because multiple substitutions at the same nucleotide position could cover the true distance between DNA sequences, making them appear artificially close to each other (Holder and Lewis 2003; Swofford et al. 1996).

- ***Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses***

Although the NJ trees are broadly used as a starting point in phylogenetic analysis, the more recommended methods in phylogenetics are those that search trees under optimality criteria such as MP (Swofford et al. 1996) and ML (Felsenstein 1981). Both methods deal with inferences about the evolutionary history of the sequences studied and assume that attributes shared among taxa come from a single ancestor (Swofford et al. 1996). Under maximum parsimony, the evolutionary history of a given data set is assessed by analysing the number of mutations that a particular tree would require to explain those data (evolutionary steps), and trees are accepted depending upon the scores (the minimum number of mutations that could possibly produce the data) (Holder and Lewis 2003).

ML is considered the most accurate method for building relationships between sequences that have been separated for a long time or that are considered to have evolved rapidly. This method takes into account multiple substitution events at the same site and considers all possible mutational pathways that are compatible with the data (see Holder and Lewis 2003; Wheland and Goldman 2001; Yang 1994, 1993). Under the ML approach a phylogenetic hypothesis is judged by how well it predicts the observed data; therefore the topology with the highest probability is considered as the best tree (Holder and Lewis 2003).

- ***Bayesian analysis***

The focus of the Bayesian inference is a quantity known as Posterior Probability (PP), which is the probability that a tree is true based on prior beliefs and likelihood (Alfaro and Holder 2006). Posterior probability integrates all possible combinations of branch length

with a substitution model for each tree. This probability is calculated using Bayes' theorem, which is the product of the likelihood of the tree (Pr[Data|tree], the probability of the data given the tree and the model) and the prior probability density of the tree and model (Pr[tree] referred to as the prior) divided by the probability of the data (Pr[data]) (Alfaro and Holder 2006; Huelsenbeck et al. 2001).

Bayes' formula: Pr[Tree| data] = Pr [Data| Tree] × Pr [Tree]/ Pr [Data]

The tree with the highest PP is chosen as the best estimate of phylogeny based on the likelihood (a measure of how well a hypothesis fits the observed data) and the prior probability, which represents the probability of a tree before the observations have been made (for a complete description of the method, see Huelsenbeck et al. 2001 and Rannala and Yang 1996).

Calculation of the probabilities requires a simulation technique known as the Markov chain Monte Carlo (MCMC), which involves two steps for phylogenetic analysis: Based on a current tree, a new one is proposed by stochastically perturbing the current tree. Then this new one is either accepted or rejected. If the new tree is accepted, then it is subject to further perturbation (Alfaro and Holder 2006). The best approximation of the PP of one tree is given by the proportion of the time that any tree is visited. When the chains converge at a stationary distribution, the tree is assumed as the best hypothesis; therefore the MCMC calculation has to run long enough to provide reliable estimates of the posterior probability and assure that the tree space have been fully explored (Huelsenbeck et al. 2001; Holder and Lewis 2003).

3.2. Phylogenetic reconstruction

In this study the four aforementioned tree-building methods were used to evaluate the position of the genus *Lagenorhynchus* in the Delphinidae family. The data from nine genes were evaluated (10420 bp), first building individual phylogenies using the NJ and MP analyses. Then the data set was divided into coding, noncoding, nuclear and mitochondrial regions. Finally all data were combined in a total-evidence phylogeny.

Combined data were evaluated using MP, ML and Bayesian analyses, and the total evidence phylogeny was constructed using the four methods.

- *NJ and MP reconstruction*

NJ trees for all genes were built using two different substitution models: the Tamura and Nei model, which accounts for different rate substitutions with $\alpha=0.5$ (as suggested by Model test; Posada and Crandall 1998), and the HKY substitution model, which takes into account the possibility of multiple mutations at each nucleotide site, transition/transversion bias and differences in nucleotide frequencies (Hasegawa et al. 1985). Nodal support was assessed using 1000 bootstrap replicates. Analyses were performed using PAUP* v. 4.0b10 (Swofford 2002) and MEGA v. 4.0 (Tamura et al. 2007).

Different strategies were used to evaluate the best hypothesis to fit the data under MP. First a weighted parsimony analysis was carried out with transversion (change from purine to pirimidine) weighted 2x transitions (changes for purine to purine or pirimidine to pirimidine) and changes in first or second codon position weighted 2x changes in third codon position. Secondly all characters were weighted equally, and indels (insertions-deletions) were first considered as a fifth state for all gene combinations and afterwards considered as missing data.

Heuristic searches are the most widely used method and allow good resolution when working with large data sets. This method uses two basic strategies: one or several trees are obtained by stepwise addition (connecting taxa one at a time), and then this tree is subjected to rearrangements to find the shorter trees (branch swapping). Here the MP analyses were performed using heuristic searches with tree bisection-reconnection branch-swapping algorithm (TBR) with 100 random stepwise additions. Bootstrap support (BP) was assessed using 1000 replicates with heuristic searches and 100 random addition sequences. Analyses were performed in PAUP* v. 4.0b10 (Swofford 2002).

Samples from *L. cruciger* and *S. coeruleoalba* were removed from the individual analyses of ACT and RAG2 genes as no sequences were available for these two species. In combined analyses, sequences for these samples were codified as missing data.

- *ML and Bayesian inference analyses*

ML analysis was performed in PAUP* v. 4.0b10 (Swofford 2002) using heuristic searching with random addition and TBR branch swapping; 100 replicates were applied and 10 trees hold in each replicate. Bootstrap support for each node was assessed using 100 replicates.

In the Bayesian analysis the MCMC searches were run with 4 chains, using 5 million generations repeated 10 times and sampling the Markov chains every 1000 generations. The number of generations discarded (burn in) was set to 200000. Bayesian phylogenetic inference was performed using Mr. Bayes v. 3.1.2. (Huelsenbeck and Ronquist 2001).

The best model to fit the data for both ML and Bayesian inference was determined with Mr. Modeltest v2.2 (Nylander 2004) using a parsimony tree as the basis for the analysis.

- *Partition Homogeneity Test (PHT) and other measurements of nodal support*

The accuracy of combining different data sets was assessed using the partition homogeneity test (PHT) (Farris et al. 1994), which tests the congruence between different partitions. The test was performed using the programme PAUP* v. 4.0b10 (Swofford 2002), using branch and bound searches with 1000 replicates.

Branch support (BS) (Bremer 1994), was calculated to measure the number of extra steps in tree lengths required to produce a tree without a particular node. The values are calculated as negative, positive or zero (Gatesy et al. 1999). Partitionate Bremer Support (PBS) (Baker and DeSalle 1997) was also calculated in order to infer the relative contribution of each data partition to each node and to detect conflict amongst data partitions. Positive values indicate support while negative values suggest conflict (Remsen

and O'Grady 2002). Both analyses were performed using 100 random addition replicates and the TBR branch swapping algorithm with the programs TreeRot v.3 (Sorenson and Franzosa 2007) and PAUP* v. 4.0b10 (Swofford 2002).

3.3. Divergence time

The time of splitting between *L. acutus* and *L. albirostris* and the time of the most recent common ancestor (TMRCA) between these two species were calculated using 1140 bp of the Cytb gene. The analysis was carried out using a Bayesian approach of the IM programme (Hey and Nielsen 2004) with the following parameters: q1=200 (q1=scalar for θ_1 maximum); m1=m2=0 (m=maximum migration rate); t=200 (t=maximum time of population splitting) -b 100000 (-b=duration of burn) -u =10 (u=generation time in years). The MCMC was implemented using 10 chains, and the program was run three times to check agreement amongst the different runs.

Different substitution rates have been previously estimated for the Cytb gene for *Lagenorhynchus* species; Cipriano (1997) gave an estimate of 0.3% MY using the rate of accumulation of transversions, while Hare et al. (2002) gave an overall estimated of 0.069% MY using all codon positions. Given that Cipriano (1997) used only transversions in his comparisons, the estimate from Hare et al. (2002) was considered the most reliable for estimating the divergence time between *L. acutus* and *L. albirostris* and for comparing these values with those obtained for other *Lagenorhynchus* species.

RESULTS

1. Sequence variation

Few indels were detected in the sequences studied. With the exception of the IRBP gene, where an insertion was detected in *D. delphis*, indels were found mainly in introns as expected: four indels were detected in CAMK, 3 indels in ACT, 2 indels in 16s rRNA gene and 20 indels in HEXB, of which 10 where found exclusively in the out-group. One SINE insertion of 107 bp with variable sites amongst the species was also detected in this gene; however the sequences of two species of *Lagenorhynchus* (*L. obliquidens and L. obscurus*) from the gene bank database and the out-group (*P. phocoena*) did not have this insertion, so it was excluded from all analyses.

Nuclear genes showed low variation, with polymorphic sites varying from 70 in HEXB gene to 8 in RAG gene. The highest numbers of variable sites were found in the Cytb gene (278) with 154 parsimony informative sites. A total of 244 phylogenetically informative sites were detected amongst all data partitions (genes) (Table II.3).

2. Phylogeny analysis

Unweighted and weighted phylogenies gave similar results for all the genes that were analyzed, showing changes only in the bootstrap support values for the different nodes. The differences in the treatment of gaps and the different distances used in the NJ analysis did not affect the topology of the trees for any of the genes that were studied; therefore only the analysis obtained using unweighted phylogenies, gaps treated as missing data, and HKY substitution model (in the NJ analysis) are presented.

Table II.3. Sequence variation and trees characteristics of individual and combined phylogenies.

Gene	Number of characters	Polymorphic sites	Informative characters	Number of trees	Tree length	CI	HI	RI
Indivdual genes								
Irbp	1103	59	7	2	64	0.9375	0.0625	0.6931
vWF	1191	45	8	11	53	0.8679	0.1321	0.5882
Lac	1058	51	16	6	58	0.8966	0.1034	0.8286
Rag	474	8	0	1	-	-	-	-
Camk	2113	52	14	12	68	0.9706	0.0294	0.923
Act	813	23	9	2	40	0.800	0.20	0.5
16s	509	36	17	16	61	0.6393	0.3607	0.2414
Cytb	1140	278	154	1	444	0.6599	0.3401	0.5424
Hexb*	1912	70	19	4	76	0.9211	0.0789	0.8636
By partition								
Nuclear	8664	331	73	3	370	0.9027	0.0973	0.7616
Coding	4966	450	185	1	652	0.704	0.2960	0.5349
Noncoding	5347	204	59	2	240	0.8625	0.1375	0.6957
Mitochondrial	1649	314	171	1	509	0.6523	0.3477	0.5330
Total evidence	10313	645	244	1	893	0.7458	0.2542	0.5717

*SINE insertion of 107 bp was excluded from HEXB gene. CI (Consistency Index) HI (Homoplasy index) RI (Retention index).

2.1. Phylogenetic reconstruction of individual genes under Maximum Parsimony (MP) and Neighbor Joining (NJ)

- **Nuclear genes**

MP analysis and NJ trees were consistent in the majority of genes evaluated, and different phylogenetic hypothesis were derived from individual genes. From the seven nuclear genes analyzed in this study, three genes had a good degree of resolution and similar topologies with both methods (HEXB, LAC and CAMK); one gene had a good degree of resolution with the NJ approach, but poor resolution at the species level with MP analysis (vWF); two genes had contrasting results when comparing the two methods (IRBP and ACT); and one gene did not show any degree of resolution with either of the methods used (RAG2).

From the first group of genes (HEXB, LAC and CAMK), only the HEXB gene recovered the relationships amongst the six species of the genus *Lagenorhynchus*, showing a monophyletic clade composed by *L. acutus* and *L. albirostris* as the sister group of a paraphyletic clade formed by *L. obliquidens, L. obscurus, C. commersoni, L. australis and L. cruciger* (bootstrap support 71 and 87%, in the NJ tree and MP tree respectively). The other two genes (LAC and CAMK) placed *L. acutus* and *L. albirostris* outside the clade formed by other delphinids. These two species form a monophyletic group in the topologies derived from the LAC gene (88 and 83% bootstrap support respectively), but their relationships remain unresolved in the topologies suggested by the CAMK gene (Figures II.3-II.5).

Although the vWF gene did not resolve the majority of relationships amongst species, at least in the MP tree, it suggested the exclusion of *L. acutus* from a weakly supported monophyletic clade composed by the other five species of the genus plus the genus *Cephalorynchus* (Figure II.6). Topologies from the IRBP, ACT and RAG2 genes are showed in Figures II.7, II.8 and II.9.
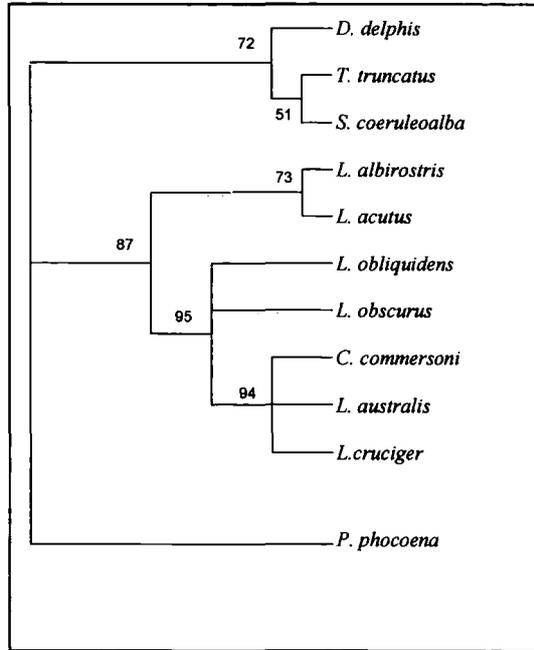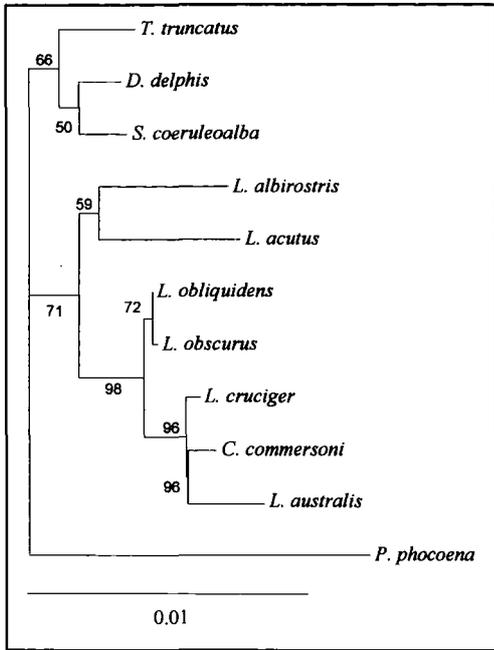
Figure II.3. Phylogenetic relationships derived from the HEXB gene. *A*. NJ tree HKY substitution model and *B*. MP consensus tree.
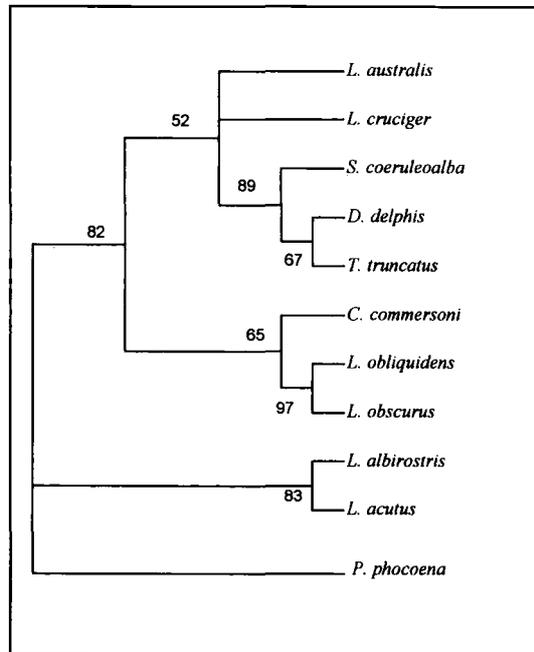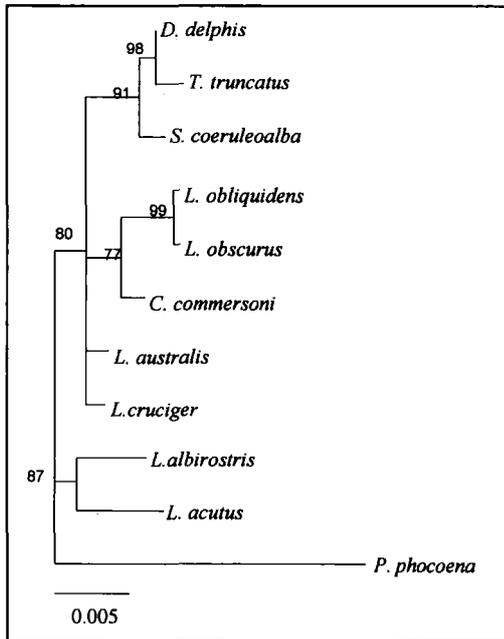
Figure II.4. Phylogenetic relationships derived from the LAC gene. *A*. NJ tree HKY substitution model and *B*. MP consensus tree.
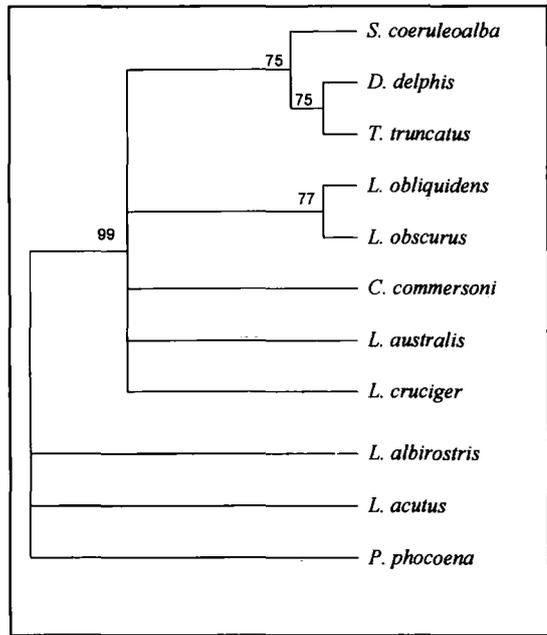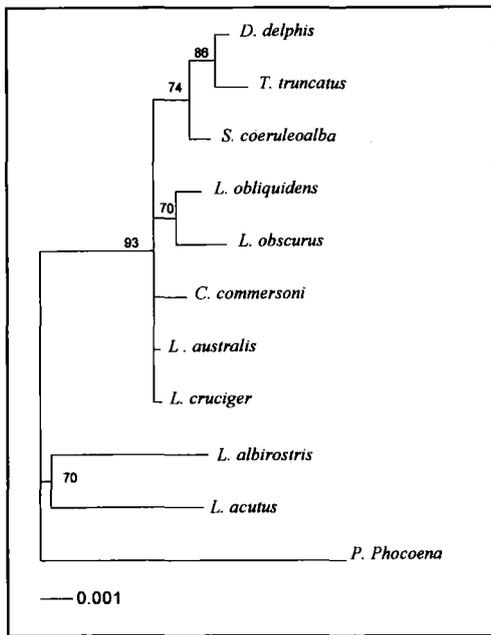
Figure II.5. Phylogenetic relationships derived from the CAMK gene. *A.* NJ tree HKY substitution model and *B.* MP consensus tree.
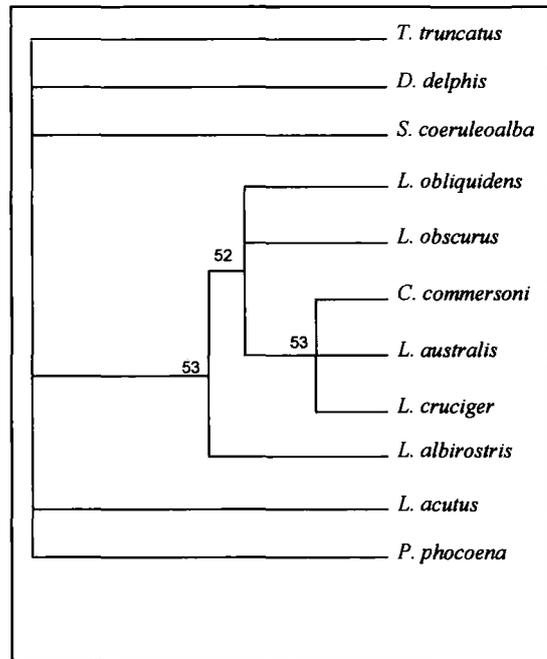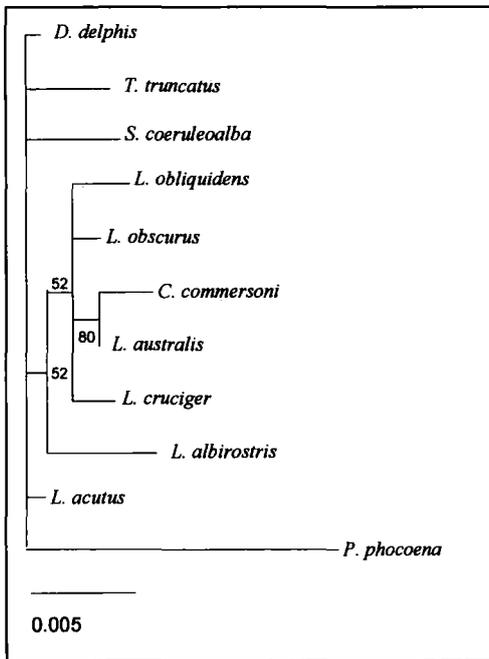
Figure II.6. Phylogenetic relationships derived from the vWF gene. *A.* NJ tree HKY substitution model and *B.* MP consensus tree.
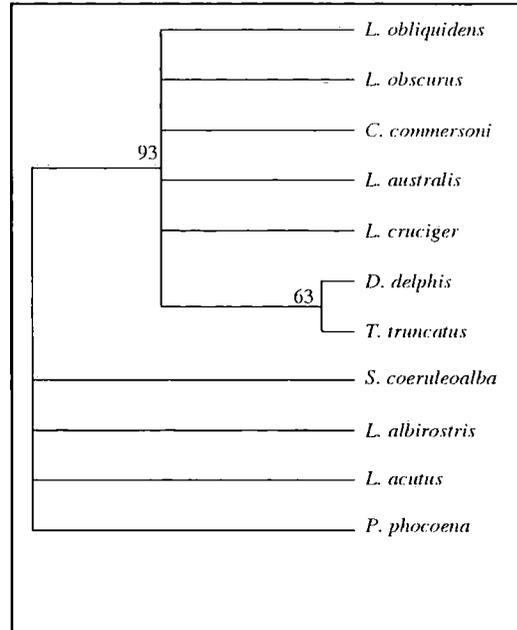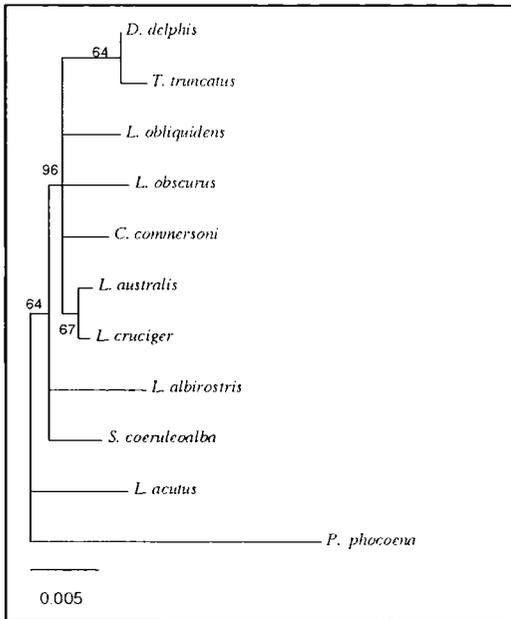
Figure II.7. Phylogenetic relationships derived from the IRBP gene. *A*. NJ tree HKY substitution model and *B*. MP consensus tree.
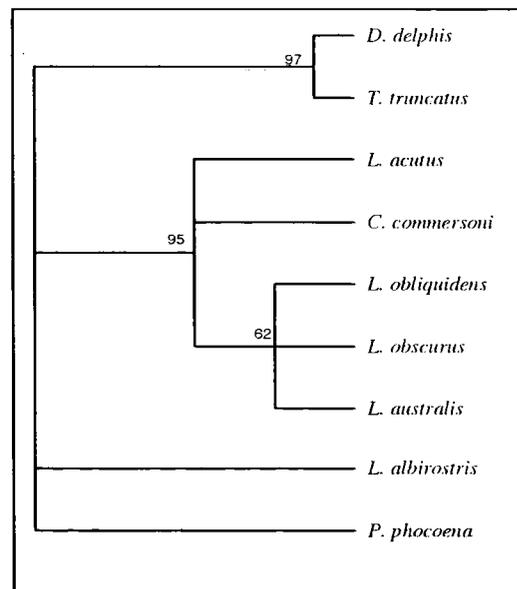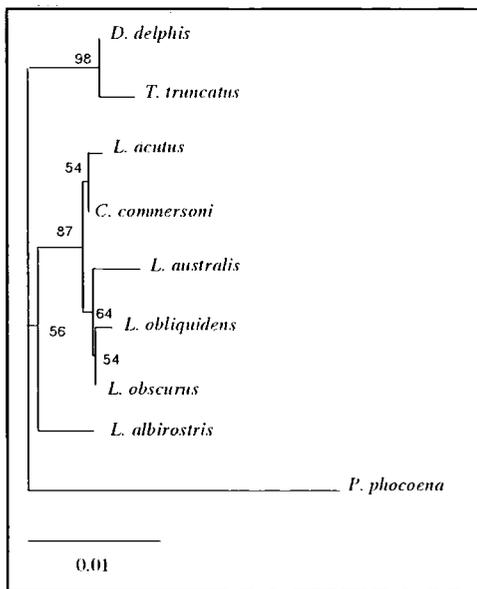
Figure II.8. Phylogenetic relationships derived from the ACT gene. *A*. NJ tree HKY substitution model and *B*. MP consensus tree.

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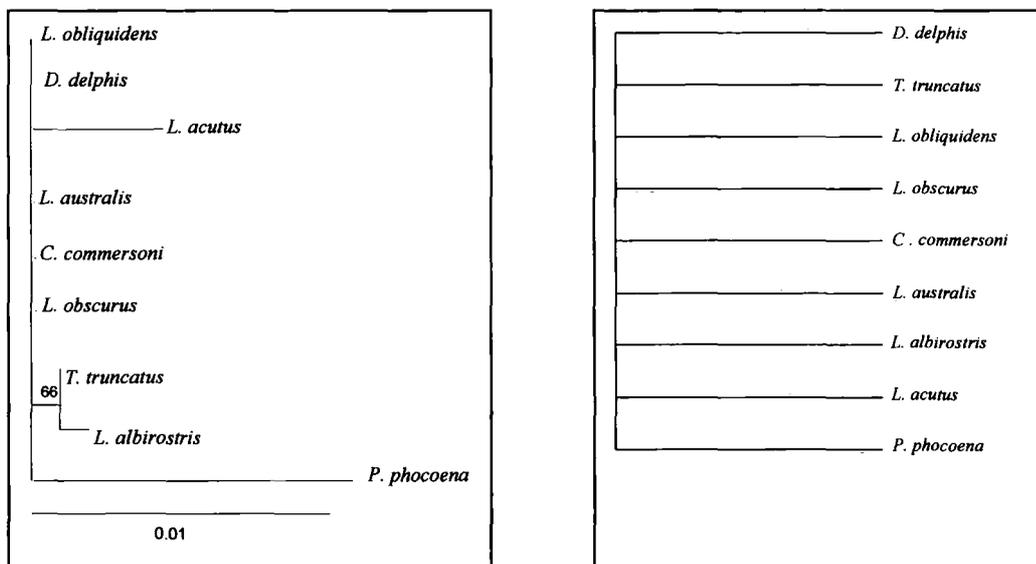
Figure II.9. Phylogenetic relationships derived from the RAG2 gene. *A.* NJ tree HKY substitution model and *B.* MP tree.

- *Mitochondrial genes*

Amongst the mitochondrial genes the only relationship that was resolved with 16s rRNA gene was the monophyly of *L. australis-L. cruciger* (97-86% bootstrap support). In contrast to the low resolution found with the 16s rRNA gene, the Cytb gene gave high resolution and agreement with the paraphyly of *L. obliquidens, L. obscurus, L. australis and L. cruciger* as reported by LeDuc et al. (1999) and as found in nuclear genes; but in contrast placed *L. acutus* and *L. albirostris* as closely related to the subfamily Delphininae (as suggested by Harlin-Cognato and Honeycutt 2006) (Figures II.10-II.11).

2.2. Combined analyses by partitions using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI)

- *Homogeneity test*

The partition homogeneity test was performed to test the congruence amongst all data partitions and between coding and noncoding regions and nuclear and mitochondrial genes. This test showed that there was no conflict amongst the different data partitions with a probability of P<0.068 in the unweighted test and P<0.140 in the weighted analysis. Homogeneity between coding and noncoding regions and between nuclear and mitochondrial genes was also accepted with probabilities of P<0.32 and P<0.50, respectively.

- *Coding and noncoding data set*

Under maximum parsimony, one more parsimonious tree was found using coding regions (Consistency index: CI 0.704). Two more parsimonious trees, differing only in the placement of *S. coeruleoalba* in the topology, were found using noncoding regions (CI 0.86 for the consensus tree). Both the coding and noncoding topologies recovered a paraphyletic group formed by *L. obscurus, L. obliquidens, C. commersoni, L. australis and L. cruciger* (Figures II.12-II.13) with a bootstrap support higher than 97%; but differed in the placement of *C. commersoni*. Both data sets excluded *L. acutus* and *L. albirostris* from the other delphinids, without resolving their relationships.
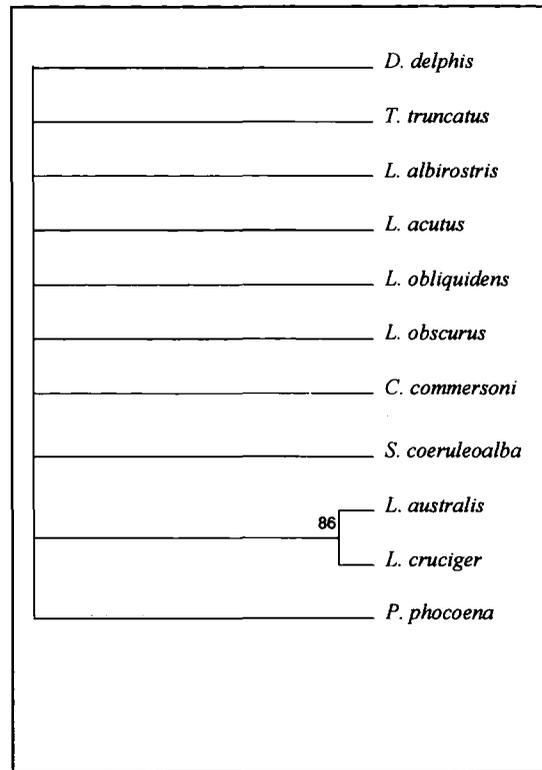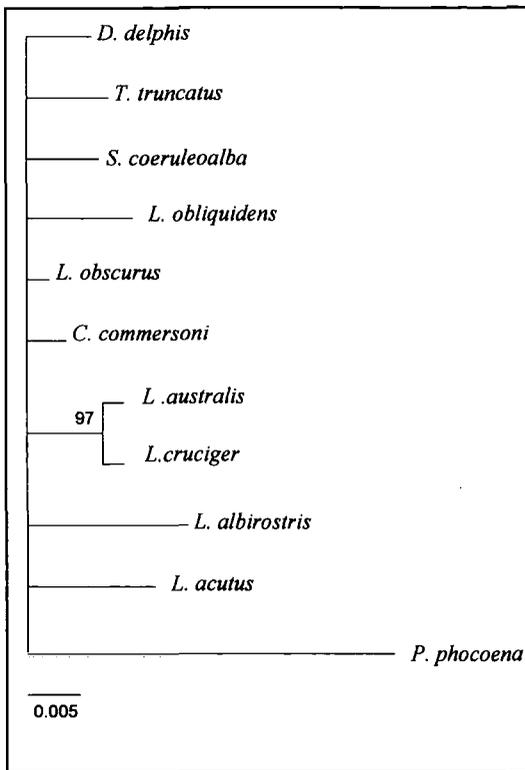
Figure II.10. Phylogenetic relationships derived from the 16s rRNAgene. *A*. NJ tree HKY substitution model and *B*. MP consensus tree.
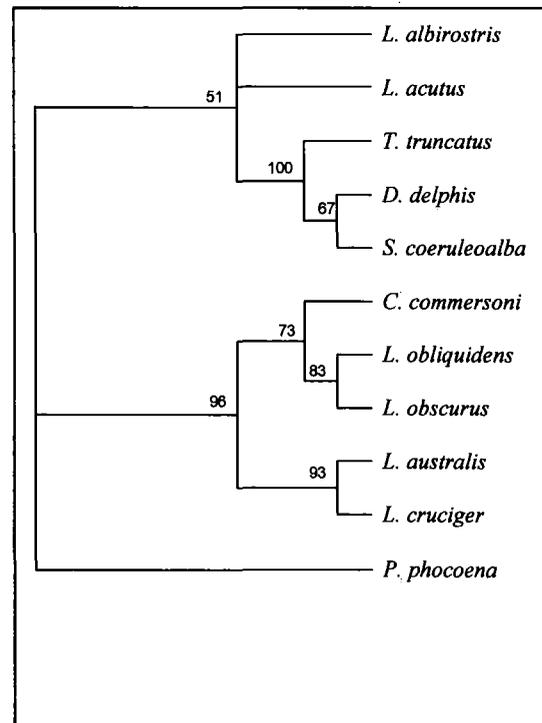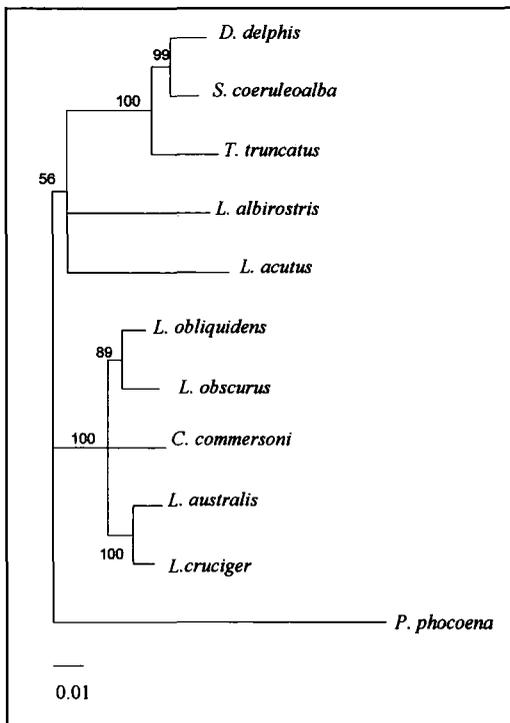
Figure II.11. Phylogenetic relationships derived from the Cytb gene. *A*. NJ tree HKY substitution model and *B*. MP tree.

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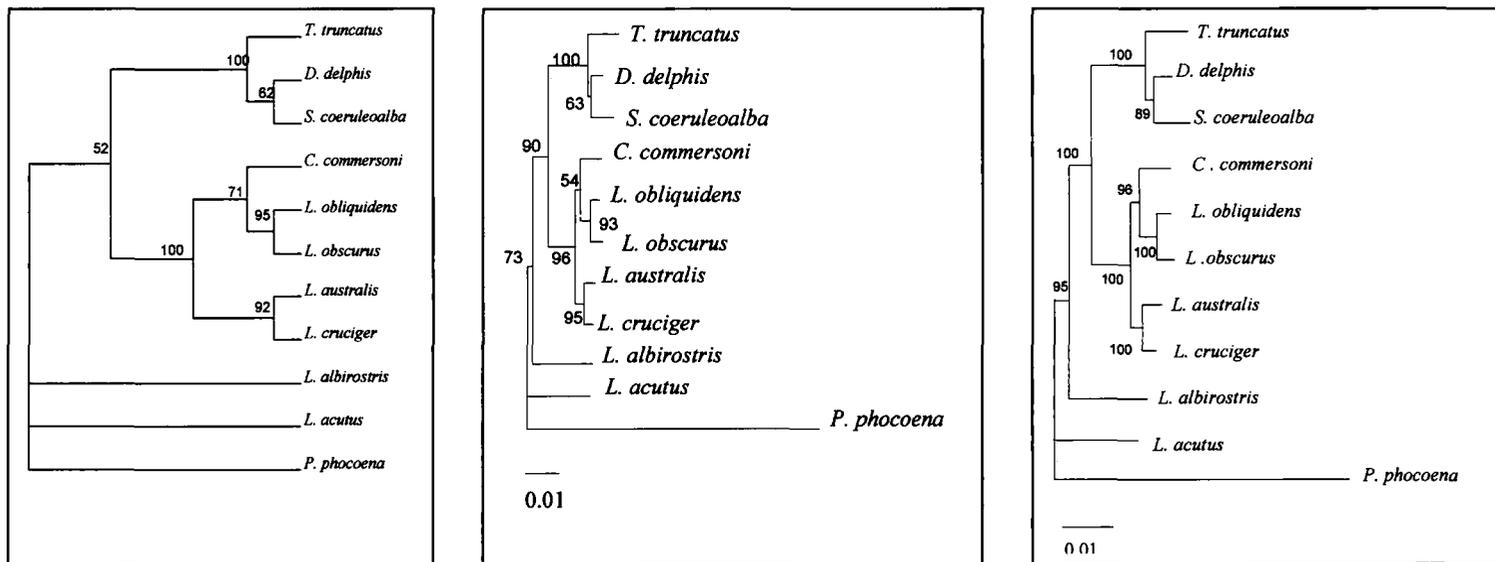
Figure II.12. Phylogenetic relationships derived from coding genes. *A*. MP tree; *B*. ML tree; *C*. Bayesian analysis tree.
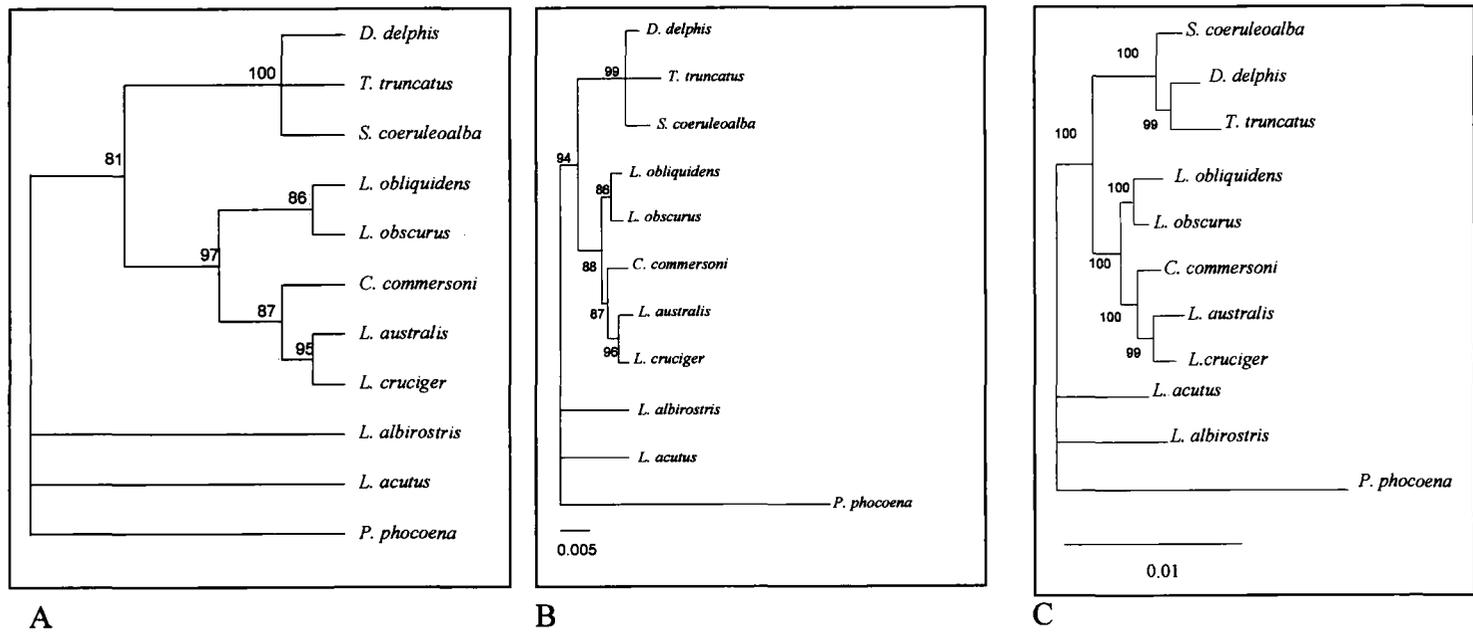
Figure II.13. Phylogenetic relationships derived from noncoding genes. *A*. MP Consensus tree; *B*. ML tree; *C*. Bayesian analysis tree.

ML and Bayesian analysis recovered topologies for both regions, similar to the ones produced by MP analysis, except for the coding region, where *L. albirostris* was joined with the other delphinids (73% bootstrap support and 95% posterior probabilities).

- ### *Nuclear vs. mitochondrial genes*

Nuclear genes showed three equally parsimonious trees differing only in the branch length and the node shared by *D. delphis, T. truncatus* and *S. coeruleoalba*. The consensus tree (CI 0.903) was similar to the one found for noncoding regions. In all analyses most relationships are stable with high bootstrap values, differing only in the relationships between *L. acutus* and *L. albirostris* (Figure II.14).

Mitochondrial genes had similar topologies under the three methods, but these topologies differed in the bootstrap support values for some nodes (see Figure II.15). In contrast with the findings using nuclear genes (Figure II.14), 16s rRNA plus Cytb data suggest the monophyly of *L. acutus and L. albirostris* and the placement of this group as a sister group of the subfamily Delphininae as suggested by the individual phylogeny of the Cytb gene although with low bootstrap support in the ML and Bayes' trees (69% and 61% respectively).

- ### *Total evidence phylogeny*

The simultaneous analysis fully resolved the phylogenetic relationship amongst all species analyzed in this study; however two different phylogenetic hypotheses were recovered with the four tree-building methods, differing in the placement of *L. acutus* and *L. albirostris*. These species were placed as a monophyletic group by NJ (bootstrap support 71%) and MP analysis (bootstrap support <50%), but not by ML and Bayesian analysis, where *L. acutus* appears to be basal (Figure II.16).

Eight nodes were recovered in all analyses, and one more parsimonious tree was found using MP (CI: 0.7458) (see Figure II.16 and Table II.3). PB support scores for each node ranged from +1 to +40, and bootstrap supports were higher than 69%,
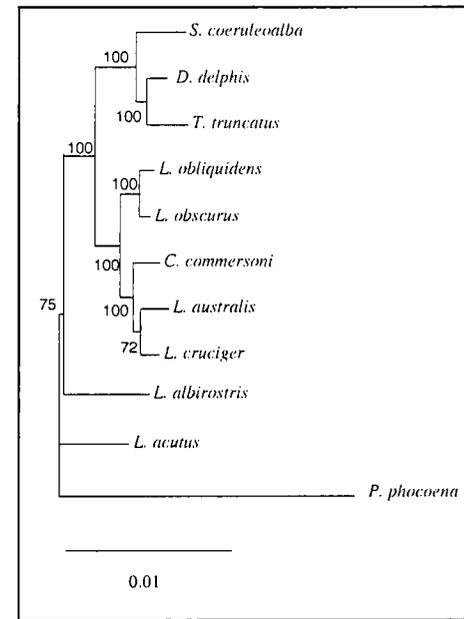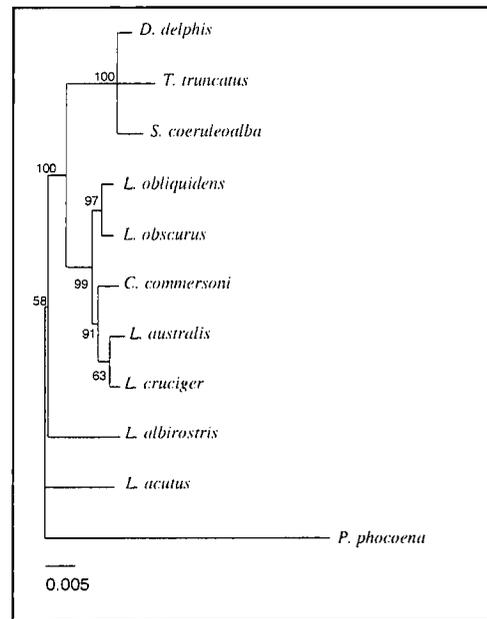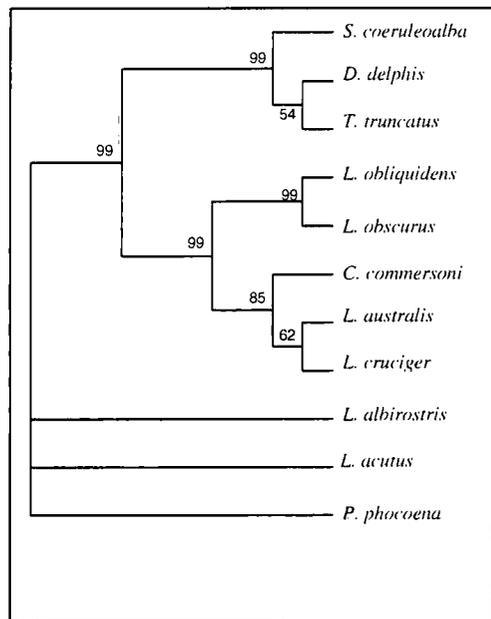
Figure II.14. Phylogenetic relationships derived from nuclear genes. *A*. MP Consensus tree; *B*. ML tree; *C*. Bayesian analysis tree.
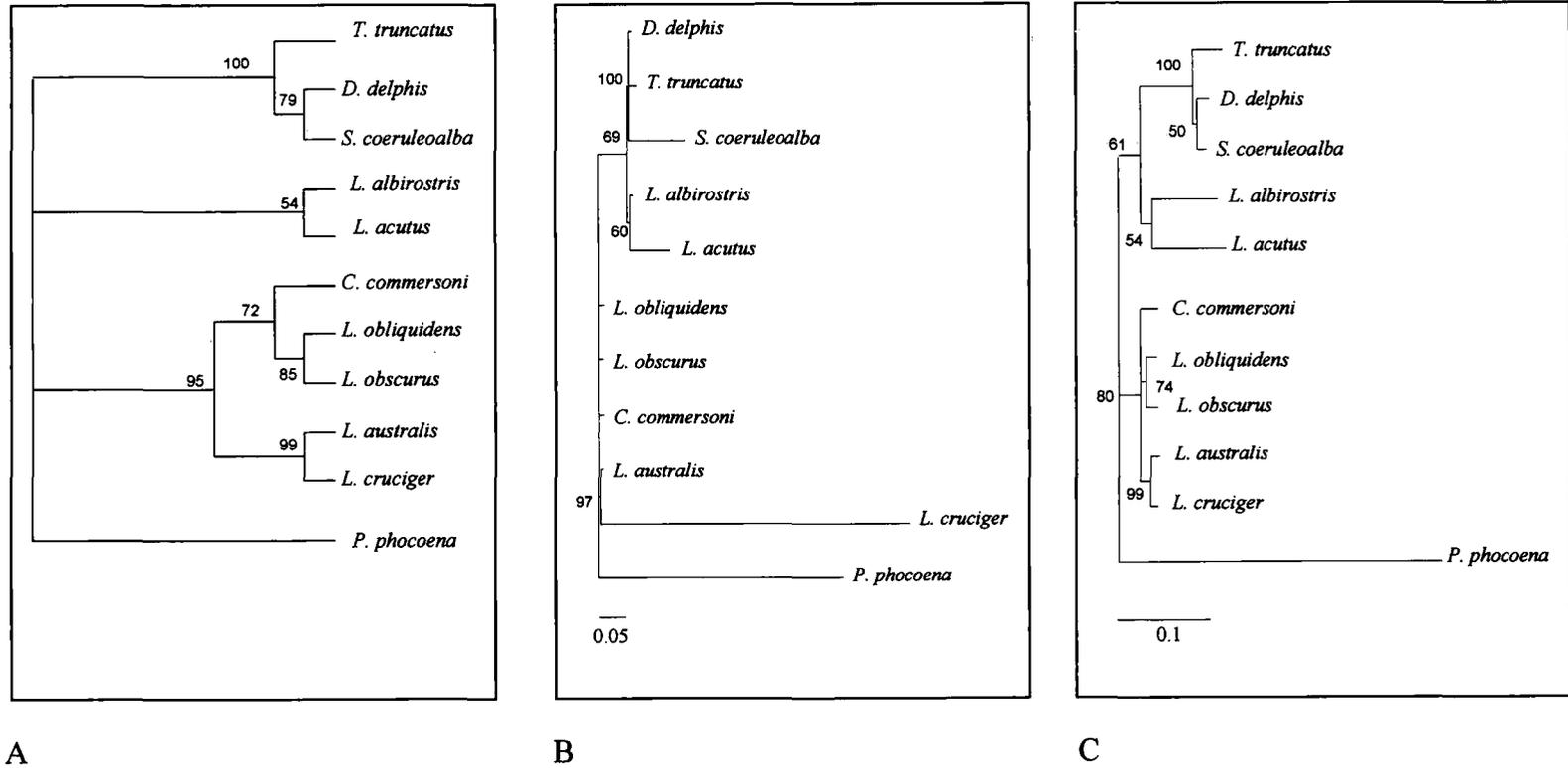
Figure II.15. Phylogenetic relationships derived from mitochondrial genes. *A*. MP tree; *B*. ML tree *C*. Bayesian analysis tree.
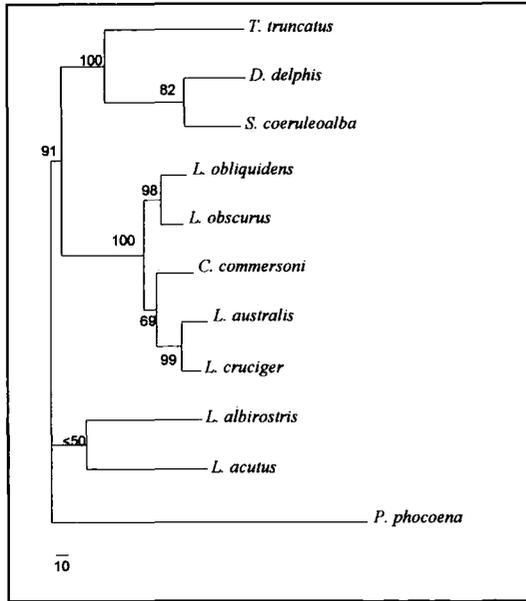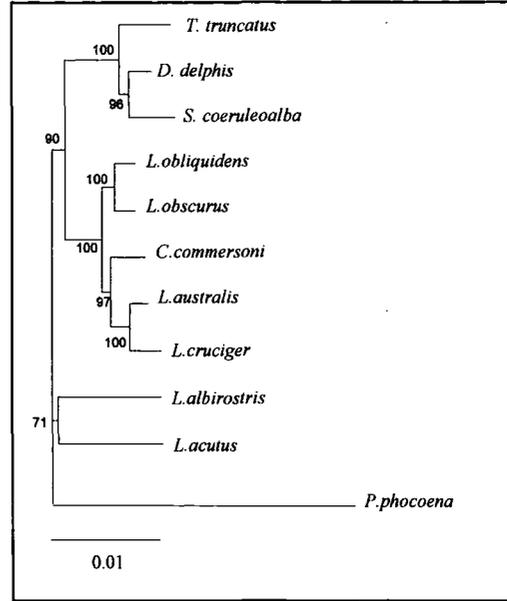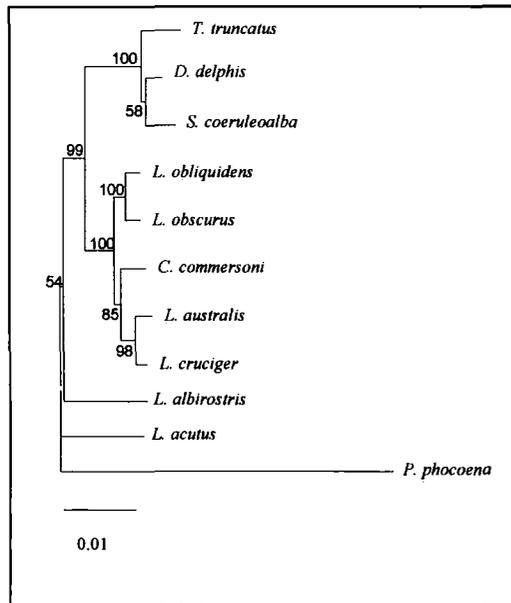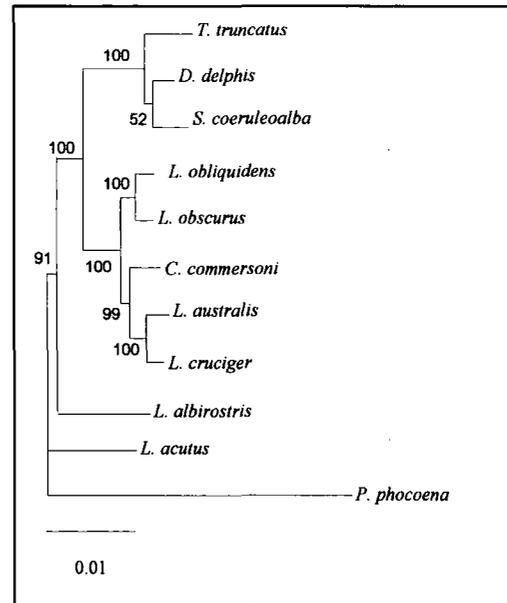
Figure II.16. Total evidence phylogeny trees derived from 7 nuclear genes and 2 mitochondrial genes. *A*. MP tree; *B*. NJ tree; *C*. ML tree; *D*. Bayesian analysis tree.

except for the node formed by *L. acutus* and *L. albirostris*. The Partitioned Bremer Support (PBS) showed that most of the support for the combined analysis comes from the Cytb gene (Table II.4). However when data were partitioned between nuclear and mitochondrial genes most of the support for the overall analysis comes from the nuclear genes (55 vs. 33, respectively). Only two genes gave support to the clade formed by *L. acutus* and *L. albirostris* (16s rRNA and LAC); the other genes gave PBS values of zero, indicating neither support nor conflict for this node (Figure II.17).

Conflict amongst data (negative PBS) was detected only in a few nodes and for a few genes. The partitions that caused most conflict with the other data for a particular node was IRBP (5 out of 8 nodes), followed by Cytb (2 out of 8 nodes). RAG and ACT genes did not provide support for any of the nodes in the analysis (PBS=0) (Table II.4).

Analyzing 2 partitions (nuclear-mitochondrial), the two data sets had a conflict in 3 of the 8 nodes: node 3 (*C. commersoni, L. australis, L. cruciger*), node 6 (*D. delphis, S. coeruleoalba*) and node 8 (see Table II.4). Nuclear genes did not provide support for clade 1 (*L. acutus, L. albirostris*).

- *Divergence time between L. acutus and L. albirostris*

The time of splitting between *L. acutus* and *L. albirostris* was calculated using the IM program (Hey and Nielsen 2004). The same distribution was obtained from different runs. The posterior distribution of t (scaled divergence time) peaked at 47.1. This value was converted to time in years using the substitution rates by Hare et al. (2002) (0.069% MY) scaled by a generation time of 10 years (Ferrero and Walker 1996) and per the number of nucleotides in this study (1140). The value obtained suggests that the divergence of these two species probably started ~6.0 MY ago. The time of the most recent common ancestor (TMRCA=51.34) predates the calculations for splitting time. Converting this estimate into time in years, using the same evolutionary rates as above, placed the most recent common ancestor between *L. acutus* and *L. albirostris* some 6.53 MY ago. These calculations are very sensitive to mutation rates; therefore care has to be taken in interpreting these results.

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Table II.4. Nodal support for the major relationships; values correspond to Figure II.15.

Node	%Bootstrap (BP)	Decay Index (BS)	Partition										
			IRBP	VWF	LAC	RAG	CAMK	ACT	16s rRNA	Cytb	HEXB	Nuclear	Mitochondrial
1	50	1	-1	0	1	0	0	0	1	0	0	0	1
2	99	9	-0.67	-0.33	1.33	0	0	0	3	3.67	1	1.33	6.67
3	66	2	-1	1	1	0	0	0	1	-3	3	4	-2
4	98	8	-0.33	0.67	3.33	0	1	0	0.33	3	0	4.67	3.33
5	100	19	3	1	3	0	7	0	-1	4	2	16	3
6	82	4	-1	0	1	0	0	0	2	2	-1	-1	4
7	100	40	0	1	5	0	8	0	1	20	5	19	21
8	91	8	3	0	1	0	7	0	0	-3	0	11	-3
total			6	4.44	14.66	0	22	0	7.33	32.67	10	55	33
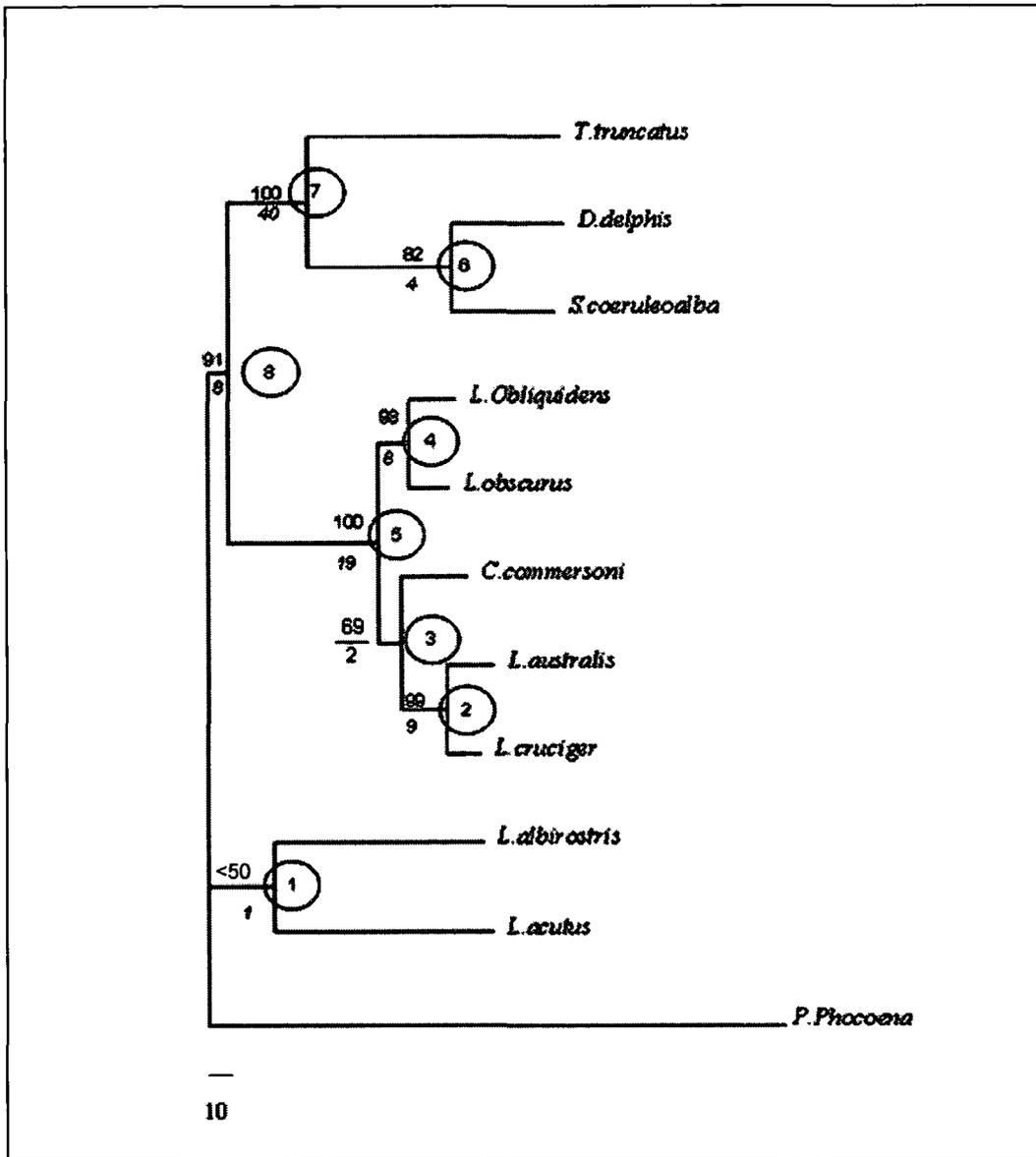
Figure II.17. Nodal supports* for the main clades obtained using the program TreeRot version 3 (Sorenson and Franzosa 2007).

*Numbers above are bootstrap support (BP) using MP and obtained with the 50% majority rule, numbers below are Bremer Support (BS). Clade numbers are indicated in circles. Note that clade 1 was not supported by ML or Bayesian analysis and was weakly supported by MP analysis (BP <50%).

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DISCUSSION

1. Phylogenetic analyses of individual genes

In this study seven nuclear genes and two mitochondrial genes were used to clarify the phylogenetic relationships amongst species of the genus *Lagenorhynchus* and its relationships with other members of the Delphinidae family. The results in this analysis showed that each gene can recover different topologies when evaluated individually, which support several authors' observations regarding the inaccuracy of using a single gene to build phylogenetic relationships amongst species (Price et al. 2005; Ballard et al. 2002; Gatesy et al. 1999; Baker and Desalle 1997).

Differences in the phylogenetic hypothesis derived from individual genes (either nuclear or mitochondrial genes) can be due to several reasons including different inheritance pathways, selection pressures or responses to evolutionary processes, hybridization between lineages, homoplasy or lineage sorting (the process whereby ancestral allelic lineages become fixed in two sister species) (Philippe and Telford 2006; Ballard and Rand 2005; Gaines et al. 2005; Hughes and Vogler 2004; Reyes et al. 2004; Shaw 2002; Grassly and Holmes 1997; Galtier and Gouy 1995). In addition, nuclear genes are expected to evolve more slowly than mitochondrial genes and amongst nuclear genes, introns are expected to accumulate mutations faster than coding genes; therefore it is expected that differences in evolutionary rates can lead to markedly different topologies (e.g., Shaw 2002; Moore 1995; Palumbi and Baker 1994).

In this study coding genes such as IRBP and vWF had few phylogenetically informative sites (8 and 7, respectively); and the phylogenies drawn from these genes were poorly resolved at the species level, something that is expected at the nuclear level in species with recent diversification (Gaines et al. 2005). Intronic sequences gave better resolution at the species level although the relationships among the species were notably different. For example, the HEXB gene (1912 bp) was the only gene to recover the monophyly of *L.*

acutus-L. albirostris and to situate this clade together with the paraphyletic group composed by the other four species of *Lagenorhynchus*; whereas the CAMK gene (2113 bp) failed to resolve the majority of relationships and placed the most conflictive lineage (*L. acutus-L. albirostris*) outside the lineage composed by the other delphinids.

2. The utility of combining data in a total evidence phylogeny

Combining data from different genes in total evidence phylogeny has been a matter of discussion for decades (Huelsenbeck et al. 1996; Miyamoto and Fitch 1995). However, many contemporaneous studies have shown that the combination of several homogeneous data sets, especially for nuclear genes, can increase the accuracy of phylogenetic hypotheses (e.g., Caballero et al. 2008; Gaines et al. 2005; Hughes and Vogler 2004). Despite the low resolution given by genes such as vWF and the contradictory results between genes such as HEXB and CAMK, this study reveals that combined data can increase the strength of some nodes and help rebuild an accurate phylogenetic hypothesis for the genus *Lagenorhynchus*.

The accuracy of combined phylogenies was assessed using the Homogeneity test, the Bremer Support test and the Partitioned Bremer Support test. All these analyses were in agreement with respect to the homogeneity of the data and the possibility of combining them in a total evidence phylogeny. The PBS analysis had positive values for the majority of nodes and genes, showing congruence amongst datasets with respect to nodes. Only 2 genes did not show any support for the nodes (RAG2 and ACT) and one gene (IRBP) showed conflict in 5 out the 8 nodes; however excluding the last gene from the total evidence analysis did not change the topology of the tree.

When the data were partitioned between nuclear and mitochondrial genes, the analysis showed that the strength in the phylogenetic signal comes mainly from the nuclear genes (see Table II.4), supporting the premise that although individual nuclear genes can resolve phylogenetic relationships only to a certain extent, together they can give a good approximation to the best phylogenetic hypothesis.

3. Phylogenetic hypotheses of the relationships of the genus *Lagenorhynchus* derived from a multilocus approach

The total evidence phylogeny presented in this study provides new insights about the relationship between *L. acutus* and *L. albirostris* and their relationships with other delphinids and strongly agrees with previous studies that consider the genus *Lagenorhynchus* to be an artificial group. The phylogenetic hypotheses suggested by the nine genes agree with the polyphyly of the genus *Lagenorhynchus* as previously suggested by Cipriano et al. (1997, using the mitochondrial control region and the Cytb gene), LeDuc et al. (1999) and May-Collado and Agnarsson (2006) using the Cytb gene, and Harlin-Cognato and Honeycutt (2006, using 2 nuclear genes ACT and RAG2 and 2 mitochondrial genes Cytb and control region), but strongly disagree with the placement of *L. acutus* and *L. albirostris* as sister taxa of Delphininae (as proposed by Harlin-Cognato and Honeycutt 2006).

3.1. The placement of *L. acutus* and *L. albirostris* in the Delphinidae phylogeny

Two phylogenetic hypotheses about the placement of *L. acutus* and *L. albirostris* in the phylogeny of delphinids were derived in this study. The first hypothesis, derived from NJ and MP analyses, suggests that *L. acutus* and *L. albirostris* form a monophyletic clade basal to all other delphinids (see Figures II.16a and II.16b). The second hypothesis, derived from ML and Bayesian analyses, proposes that *L. acutus* and *L. albirostris* are not closely related to each other and that *L. acutus* is basal in the phylogeny of delphinids (see Figures II.16c and II.16d).

The latter hypothesis should be preferred for three reasons: the monophyly of *L. acutus* and *L. albirostris* was supported by only small bootstrap values in the MP tree (less than 50%). Secondly, it is well known that MP analysis, but not ML analysis, can be affected by long-branch attraction, where two branches that are not adjacent on the true tree are inferred to be the closest relatives of each other (Holder and Lewis 2003). Finally, ML and Bayesian analyses take into account all possible evolutionary pathways and are therefore

expected to be a better approximation of the true phylogeny. Although these findings have to be considered as only two of the possible hypotheses for explaining the relationships in this genus, this study has given new insight into the phylogenetics of delphinids and suggests that *L. acutus* and *L. albirostris* should probably be placed in a new subfamily as proposed by LeDuc et al. (1999) and in separate genera. LeDuc et al.'s proposal for changing the name of *L. acutus* to *Leucopleurus acutus* and retaining the name *Lagenorhynchus* for *L. albirostris* is supported by this study.

3.2. Paraphyly of Lagenorhynchus within the new Lissodelphininae subfamily

This study agrees with previous studies (Harlin-Cognato and Honeycutt 2006; May-Collado and Agnarsson 2006; LeDuc et al. 1999) on the paraphyly of *L. obscurus*, *L. obliquidens*, *L. australis and L. cruciger* and resolves the monophyletic relationships between *L. obliquidens–L. obscurus* (100% bootstrap support) and *L. australis-L. cruciger* (98-100% bootstrap support). The monophyly of *L. obliquidens-L. obscurus* has been broadly discussed and analyzed by several authors (e.g., Cipriano 1997; Hare et al. 2002), and this strongly corroborates the relationships between these two species. This study also agrees with the proposal by May-Collado and Agnarsson (2006) that *L. australis* and *L. cruciger* are more closely related to *C. commersoni* than to the other two species (*L. obliquidens-L. obscurus*) with a bootstrap support ranging from 69-99%. However these relationships deserve further analyses and the inclusion of nuclear genes for all species of the genus *Cephalorhynchus* will be necessary in order to have a better picture about the relationships between *Cephalorhynchus* and *Lagenorhynchus* genera.

4. Interpretations of the demographic history of the genus Lagenorhynchus

The species of the genus *Lagenorhynchus* as classified to date, have a markedly antitropical distribution, with two unique species distributed in the North Atlantic (*L. acutus* and *L. albirostris*), one in the North Pacific (*L. obliquidens*), and three in the southern hemisphere (*L. obscurus, L. cruciger* and *L. australis*). Only a few morphological characteristics have been found to distinguish these six species; they were grouped together based mainly on similarities in colour pattern and soft-facial morphology

(Mitchell 1970; Fraser 1966). Miyasaki and Shikano (1997), studying the differences in skull morphology of the six species, found that they can be distinguished by only eight cranial characters. For example they found that *L. albirostris* is distinguishable from the other five species by only five characters and *L. acutus* by three. *L. obliquidens* and *L. obscurus* were separated from *L. australis* and *L. cruciger* by four characters; whilst both of the former two and the latter two species were separated by only two characters. These few differences have supported the classification of these six species into a single genus with antitropical distribution, however the new classifications, based in molecular data has generated controversies about the validity of the genus (see previous section).

The pattern of antitropical distribution has been found in several marine taxa such as phocoenids, whales, elephant seals, invertebrates and fish (see review in Lindberg 1991). It has been explained as the consequence of several palaeoclimatic changes that have occurred during different geological eras; for instance, the increase of the upwelling regimen during the middle Miocene (12-15 MY ago); the reduction of upwelling and changes in sea temperatures during the Pliocene (5.3 – 1.8 MY ago); and changes in current patterns and the glaciations of the northern hemisphere in the Pleistocene (~1.8-0.01MY ago) (Fajardo-Mellor et al. 2006).

No agreement has been found to explain the speciation and antitropical distribution of *Lagenorhynchus* species with the aforementioned events. For instance, Cipriano (1997) in a preliminary study including four of the six species of the genus (*L. acutus, L. albirostris, L. obscurus* and *L. obliquidens*) suggested that *L. acutus* and *L. albirostris* probably started to diverge close after the radiation of dolphins approximately ~11 MY (late Miocene; see Barnes 1990 and Fordyce and Barnes 1994), and therefore the hypothesis of the origin of these species as a result of the transgression of the tropical barrier during the Plio-Pleistocene (see phylogeography section in chapter I) could not be accurate. This author was the first to suggest that the placement of the six species in a unique genus with antitropical distribution could be erroneous.

The main finding in this study is that the position of *L. acutus* and *L. albirostris* is basal in the phylogeny of Delphinidae. The time of the most recent common ancestor between *L. acutus* and *L. albirostris* (6.53 MY ago) and the time of the divergence between these two species (~6.0 MY ago), are in agreement with earlier suggestions about the polyphyly of the genus *Lagenorhynchus* (e.g., Cipriano 1997; LeDuc et al. 1999). It has been accepted that the polyphyly found in many species can be the cause of inaccurate species delimitation "when the described phenotypic boundaries of nominal species do not accurately reflect the history of population differentiation and speciation" (Funk and Omland 2003 p. 405). Moreover, it is also possible that the speciation of these taxa took place during a long period of genetic isolation causing deep molecular divergences at the nuclear level with little morphological differentiation, as has been suggested for other species; e.g., right whale species (Gaines et al. 2005).

Fordyce and Barnes (1994) suggest that "Changes in diversity at genus and species level potentially reveal links between cetacean evolution and geological events." Therefore a possible hypothesis should consider the analysis of the phylogenetic relationships among the species, the time of the most recent common ancestor, and the study of the geological event that could have affected the evolutionary history of dolphins and their past dispersion routes. As stated before, the total evidence analysis in this study placed *L. acutus* and *L. albirostris* as the basal species of delphinids, with *L. albirostris* being most closely related to the other *Lagenorhynchus* species. The lack of strong support for monophyly between *L. acutus* and *L. albirostris* also suggests that *L. acutus* is probably basal (or retains the characteristics of the ancestral species) of the other delphinids.

4.1. Hypothesis about dispersion and speciation in the current genus Lagenorhynchus

A possible hypothesis for explaining the findings in this study is that *L. acutus* and *L. albirostris* originated in the North Atlantic, with the most recent common ancestor living in this basin approximately 6.53 MY ago during the Late Miocene- early Pliocene (as suggested by the TMRCA values). Although most of the studies placed the dispersion of marine taxa from the Pacific towards the Atlantic (e.g., Pastene et al. 2007; Fajardo-Mellor et al. 2006; Briggs 2003), it is plausible that the ancestor of *Lagenorhynchus*

dispersed into the Pacific via the Bering strait (North) or via the Panamic sea way (South), subsequently giving origin to *L. obliquidens* (the only species distributed in the North Pacific and with a broad distribution range from temperate regions to cool waters) by vicariant speciation. This hypothesis is supported by the position of *L. acutus* and *L. albirostris* in the total evidence tree (see Figures II.16c and II.16d) and by the finding in this study that the centre of origin for both species is probably the western North Atlantic (see chapters III and IV).

- *Dispersion route 1: The Bering Strait*

This hypothesis is supported by recent studies and stratigraphic information that suggest that the Bering Strait was historically open with a minimum age ranging from 4.8 to 7.3 ± 7.4 MY ago (Marincovich and Gladenkov 1999). Gladenkov et al. (2002) also date the time of opening this strait (based on the migration of the marine bivalve mollusk Astarte from the Arctic into the North Pacific) at the end of the Miocene, approximately 5.32 MY ago. Moreover, Briggs (2003) and Vermeij (1991) stated that by approximately 3.5 MY ago the Bering Strait apparently allowed an unrestricted migration between the Arctic-North Atlantic and the North Pacific.

Studies that support the hypothesis of migration of fauna via the Bering Strait have focused mainly on mollusk taxa and predict that this migration has been mainly from the Pacific towards the North Atlantic, however several examples have shown that migration in the other direction may have also been possible (see Briggs 2003). One well-known case is the Atlantic cod (*Gadus morhua*) and the Pacific cod (*Gadus macrocephalus*), which are descended from an Atlantic lineage that invaded the Pacific in two waves, one at least 3.5 MY ago and the other approximately 2 MY ago when the Bering Strait was open and the Arctic was ice free (Briggs 2003; Raymo et al. 1990). Vicariant speciation in *Lagenorhynchus* from an Atlantic ancestor likely took place later after the establishment of the Bering Strait, as hypothesized for other marine taxa, in response to the intermittent closure and opening of this Strait during glaciated epochs (see Lindstrom 2001; Lindberg 1991).

- *Dispersion route 2: The Panamic sea way*

An alternative hypothesis for explaining the presence of *L. obliquidens* in the North Pacific is the dispersion of the ancestral lineage of North Atlantic origin (which as stated before probably inhabited this basin around 6.53 MY ago during the Miocene-early Pliocene periods) towards the North Pacific before the closure of the Panamanian seaway, which occurred approximately 3.1 MY ago in the Pliocene (Lindberg 1991; Fordyce 1989). An interchange via the Panamanian seaway is possible because, as stated by Lindberg (1991, p. 308), "many of the well known and well documented cases of discontinuous distribution of marine taxa involve east-west interchanges or separations," and these interchanges between the North Atlantic and North Pacific were common before the closure of the Panamic portal (Lindberg 1991).

This hypothesis would, however, require that the ancestral species has a different range of distribution than exhibited today for its descendants *L. acutus* and *L. albirostris*, which are restricted to temperate and sub-polar waters. Migration toward the South could be explained by the changes in distribution and dispersal patterns observed in other marine taxa, which have been correlated with the continuous changes in sea temperatures, current patterns and sea productivity (i.e. upwelling) during the Miocene-early Pliocene epochs (e.g., Pastene et al. 2007; Matul and Abelmann 2005; Wares 2002).

Migration and dispersion patterns of marine taxa during the Miocene and Pliocene epochs have focused mainly in invertebrates' species. For example, Vermeij (2005) examined the fossil record of several gastropod clades and two bivalve clades in order to understand the migration pattern between two marine provinces in the Atlantic Ocean; the Caloosahatchian and Gatunian provinces. The latter province refers to biota from tropical America, south of the Gulf of Mexico and Florida; whilst the former refers to northern coastal habitats (Vermeij 2005; Figure 1). This author found that about 31% of subgenus- and species-level taxa migrated toward the Gatunian province during the late Miocene, but no taxa migrated northward to the Caloosahatchian Province. The pattern was the opposite during the Pliocene until the early Pleistocene epoch when 40 taxa migrated north, but only 4 taxa migrated toward the Gatunian province. This differential migration between

provinces during different periods has been explained in terms of the changes in the sea conditions and pattern of ocean circulation (beginning in the middle Miocene).

Dolphins are endothermic animals, highly mobile and with large bodies, and therefore require large amounts of food to maintain an internal body temperature higher than the environment (Doksæter et al. 2008). These characteristics have led to the hypothesis that prey distribution and abundance, are the key factors that have guided their distribution and evolution. Along these lines, Berger (2007) discussed the evolution of toothed and baleen whales in terms of seasonal food floods and upwelling patterns, based on the theory that silicate-supported upwelling (linked to high productivity) and deep mixing within the southern ocean were the main forces driving the evolution of cetaceans.

Paleoceanographic studies of Neogene sediments have suggested that the primary productivity in oceans during the latest Miocene-early Pliocene epochs was localized in upwelling zones in the North and West Indian Ocean and the North Pacific Ocean (Dickens and Owen 1999). Other zones have also been suggested recently in the Atlantic Ocean, where a bimodal pattern of high productivity peaks have been found (see Hermoyian and Owen 2001). In agreement with Berger (2007), before the late-Pliocene the subtropical upwelling regions were the main source of abundant food for cetaceans and the dispersion and distribution of many species in this regions were guided by the high predictability of these productivity zones. Berger's hypothesis supports the suggestion that ancestral populations of *L. acutus* and *L. albirostris* probably migrated to the South and West — via the Panamic portal before its closure (3.1 MY ago) — during the Miocene-early Pliocene epochs, guided by the availability of food in southern tropical areas, this could have resulted in the divergence of the North Pacific migrants from populations that remained in the North Atlantic.

It should be noted that other events such as the glaciations in the Northern Hemisphere have also been claimed as being responsible for the dispersion and shifting of the species south of their original geographic range (see Wares 2002). Nevertheless, glaciated epochs as the cause of the South and West migration of the ancestral species that gave origin to *L.*

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obliquidens can be ruled out here as some authors have argued that the first Pliocene cooling episode took place between 2.9 and 2.4 MY ago (Briggs 2003; Raymo 1994), soon after the closure of the Panamic portal.

- **Speciation in the North Atlantic (*L. acutus-L. albirostris*) and the North Pacific (*L. obliquidens*)**

It is possible that populations of the ancestral species of *Lagenorhynchus* that were once distributed in southern tropical areas began a new migration toward high latitudes in the late Pliocene epoch when "subtropical upwelling regions lost some of their appeal as sources of superabundant food" (Berger 2007, p. 2406). In agreement with Berger (2007), the extensive high-latitude migrations of marine mammals during the late Pliocene were a response to the lack of predictability of low-latitude feeding zones, in contrast with the favourable conditions in high latitudes. This author also suggested that populations that selected different migration routes no longer met and consequently began to diverge; "the fact that they diverged so little is an evidence for the youth of the origin of these forced migrations" (Berger 2007, p. 2414).

L. acutus and *L. albirostris* probably diverged by allopatric speciation, followed by secondary contact or by sympatric speciation (see examples in Stefanni and Knutsen 2007), promoted by the conditions of the North Atlantic seas during glaciated epochs and the selection of different habitats (e.g., *L. acutus* prefers offshore habitats, whilst *L. albirostris* is restricted to coastal regions; see next section). The molecular divergences between these two species could have been accelerated due to the loss of ancestral alleles caused by strong and perhaps long periods of bottlenecks as occurred in other species (see reviews in Hewitt 2000, 1996), promoted by the fragmentation or loss of marine habitats (see Wares 2002; Carstens and Knowles 2007; and also discussions in chapters III and IV).

Following the aforementioned hypotheses and the previous findings of Harlin-Cognato et al. (2007) and Hare et al. (2002), ancestral individuals of *L. obliquidens* are suggested to have expanded and evolved in the North Pacific and colonized the South Pacific in a

North-South dispersal event. This dispersion North-South was first suggested by Hare et al. (1997) who using 5 independent loci, found that individuals of *L. obliquidens* and *L. obscurus* form a paraphyletic clade (Hare et al. 1997; Figure 3), suggesting that *L. obliquidens* gave origin to *L. obscurus* and that the dispersion and colonization probably took place during the Pleistocene with both species isolated by approximately 0.74-1.05 MY. In contrast, Harlin-Cognato et al. (2007) using Bayesian analysis of mitochondrial DNA sequences suggested that the divergence between these two species is much older. Thus they proposed that the North-South equatorial transgression occurred approximately 2 MY ago [95% CI: 1.3–2.9 MY ago] at the end of the cooling period during the Plio-pleistocene epoch. In agreement with these authors, the temporal disruption of the tropical barrier during this period, combined with the novel distribution and abundance of prey (e.g., anchovy) in the tropical Pacific seems to have played an important role in the dispersion of *L. obliquidens* towards the South-Pacific. The subsequent changes in sea temperatures and the decline in productivity (affecting prey availability and distribution), seems to have lead to the isolation of a founder population of *L. obliquidens* in a refugial zone in the cool waters of the South Pacific/Indian Ocean, which may have led to the origin of *L. obscurus* (Harlin-Cognato et al. 2007; see also Briggs 2003 and Berger 2007).

L. obscurus in the phylogenetic analyses performed in this study (see Figure II.16) consistently shares a lineage with *L. obliquidens,* supporting the putative ancestral connection. The relationship between the three species in the Southern Hemisphere is less clear. *L. australis and L. cruciger* are consistently within a lineage that also includes *Cephalorhynchus* (for trees based on the selected species that have been included in this study). May Collado and Agnarsson (2006) suggested that these two species should be placed in the genus *Cephalorynchus*; however although the data in this study, using nuclear genes, show evidence for a reclassification of these species, this finding should be interpreted with caution. Given that only one species of the genus *Cephalorhynchus* was included in this analysis; further analyses involving all species in both putative genera will be required.

CHAPTER III: PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE WHITE-SIDED DOLPHIN (*LAGENORHYNCHUS ACUTUS*)
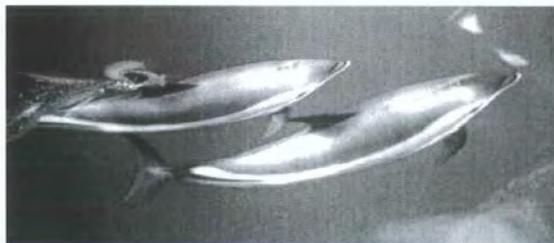
INTRODUCTION

1. Distribution and Phylogeography of L. acutus in the North Atlantic

The Atlantic white-sided dolphin (*Lagenorhynchus acutus*, Gray 1828) is a pelagic species, endemic in the North Atlantic, with a preference for temperate and sub-polar waters (Weinrich et al. 2001; Evans 1992; Mikkelsen and Lund 1994) (refer to Figure II.1). Its geographic range extends from New England to West Greenland in the western North Atlantic and from East Greenland, Iceland, British Isles, the North Sea and Norway in the eastern North Atlantic (Gaskin 1992).

In the western North Atlantic the species inhabits waters from central West Greenland to North Carolina (about 35°N) (Hamazaki 2002; Evans 1987). In the eastern North Atlantic it is commonly distributed from Iceland and the western Barents Sea, south to the Bay of Biscay, being more abundant at 56°N than south of this latitude (Evans 1992). In the British Isles, its distribution is concentrated around the Hebrides, northern Isles and northern North Sea in waters 100-500 m deep. It also occurs regularly off western Ireland, but has not been reported frequently in the Irish Sea and is considered very rare in the southern North Sea (Reeves et al. 1999; Evans 1991; Evans 1992; Gaskin 1992) (Figure III.1).

Although the general distribution of *L. acutus* has been well studied, few studies have been published about their seasonal migration and spatial and temporal distribution in specific areas (e.g., MacLeod et al. 2007; Weir et al. 2007; Waring et al. 2006, 2007; Hammond et al. 2002; Weir et al. 2001; Northridge et al. 1997; Sergeant et al. 1980). In Scotland the species prefers areas with temperatures >12.2°C more than colder waters and has been mainly recorded at distances from 36-96 km from the coast (MacLeod et al. 2007). In the western North Atlantic it has been categorized as a near-shore species commonly reported in waters between 10-15°C and depths from 400-500 m (Hamazaki 2002; Reeves et al. 1999).
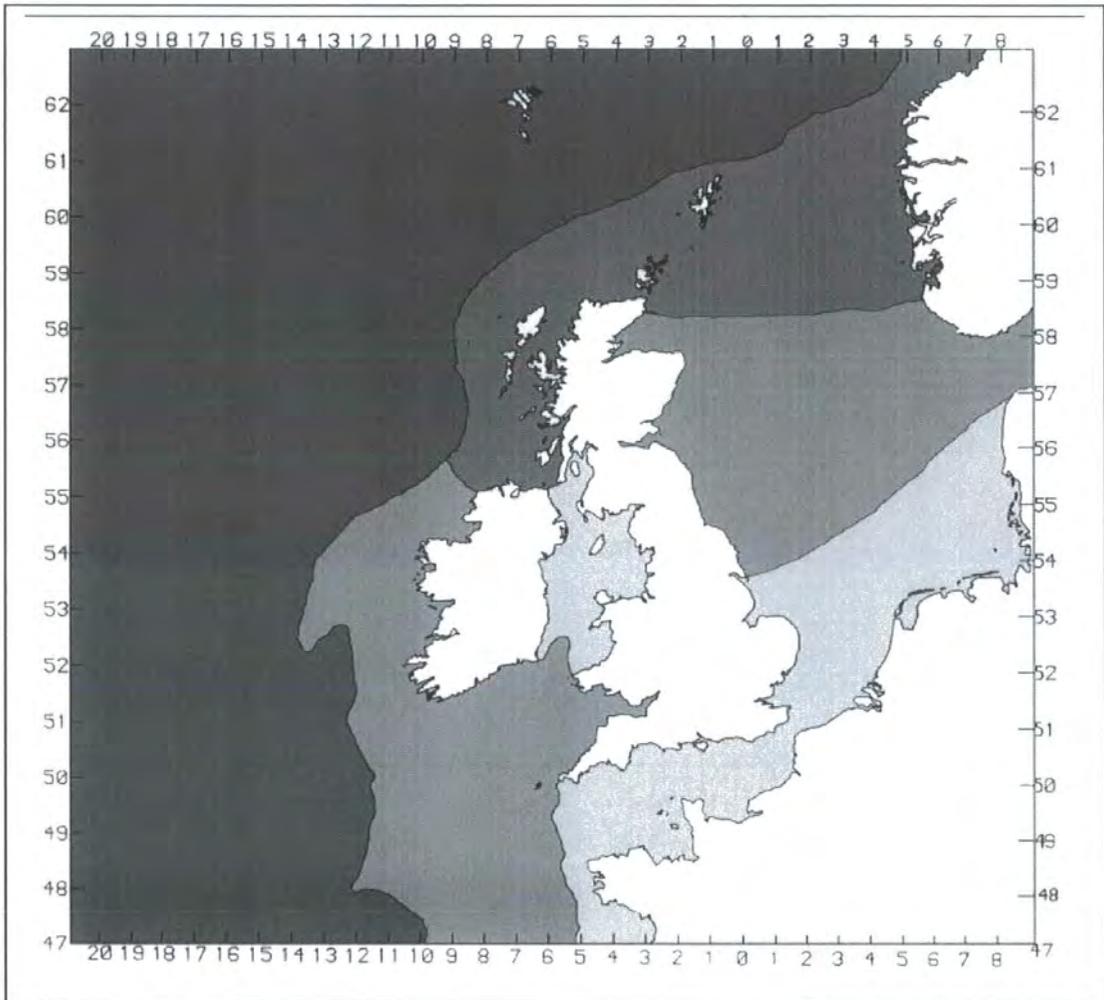
Figure III.1. Distribution of the Atlantic white-sided dolphin (*Lagenorhynchus acutus*) in UK and adjacent northern European waters.

Source: Sea Watch Foundation data and knowledge on the ecology of the species. Courtesy Professor Peter Evans from Sea Watch Foundation. Status: Regular, common or fairly common (dark shading); Regular, uncommon (fairly dark shading); Occasional (intermediate shading); Casual/Absent (light shading).

Studies on terrestrial and fresh water organisms have shown that populations distributed in the highest latitudes frequently have a lower degree of genetic diversity than species distributed in lower latitudes; i.e. the tropics (Hewitt 2001). This pattern has been attributed to climatic and environmental changes in the northern hemisphere during the ice ages, which generate the isolation of populations in refugees and their subsequent and rapid increment in population sizes due to the colonization of new, unoccupied habitats after the retreat of the ice (Hewitt 2000, 1999, 1996; Stanley et al. 1996). The effects of these processes —reduction of population sizes, colonization and expansion— in the DNA of the organisms have been well documented (Excoffier 2004; Schneider and Excoffier 1999; Harpending et al. 1998; Harpending 1994; Rogers and Harpending 1992), and the statistical theories associated with demographic expansion have become of popular use to address hypotheses about the time of the expansion and the effective sizes of populations before and after the expansion (e.g., Pastene et al. 2007; Palo 2003; Hare et al. 2002).

As reported for other species of marine mammals (e.g., Pastene et al. 2007; Palo and Vainola 2006; Palo 2003; Palo et al. 2003), it is possible that the spatial distribution of lineages, demography history and genetic diversity of L .$acutus$ populations were affected by the glacial cycles in the North Atlantic. If this is so, a loss of allelic diversity will be expected in this species due to genetic drift in bottlenecked refugia, and a clear unimodal shape in the distribution of pairwise differences resulting from range expansions in warmer periods. In this chapter I will test this hypothesis using different coalescence approaches, the characteristics of the distribution of pairwise differences (mismatch distribution) and the sensitivity of the neutral test for detecting demographic changes.

2. Population structure

Knowledge about population structure of L. $acutus$ is limited (e.g., Weir et al. 2007; MacLeod et al. 2007; Waring et al. 2007; Hammond et al. 2002; Weir et al. 2001; Northridge et al. 1997). Studies based mainly on sightings; strandings and incidental takes have suggested the existence of three population stocks in the western North Atlantic: Gulf of Maine, Gulf of St. Lawrence and Labrador Sea. This division was proposed by Palka et al. (1997) primarily for the absence of sightings of L. $acutus$ between the first two regions.

However the existence of a separate stock in the Gulf of Maine is not clear and remain uncertain, for example Weinrich et al. (2001) found no evidence to consider this group as a resident population based on data collected from commercial whale-watching vessels and dedicated platforms in the southern Gulf of Maine from 1984-1997. The population structure is also poorly understood in the eastern North Atlantic, where studies about the species have been based mostly on opportunistic surveys and only a few long-term surveys (e.g., Evans and Hammond 2004; Hammond et al. 2002).

Mikkelsen and Lund (1994) suggested the existence of a single population of this species across its geographic range, based on phenotypic differences among skulls of 228 specimens of *L. acutus* from the eastern and western North Atlantic. In contrast to the suggestion by Palka et al. (1997), these authors did not find evidence to subdivide populations into a northern and southern stock in either of the two areas studied or into the western and eastern North Atlantic.

The identification of population boundaries in *L. acutus* is complicated by the difficulties in carrying out long-term studies and the constrictions imposed by the habitat and behaviour of marine species. Thus the availability of samples from stranded animal and biopsies of free living animals are an important resource of genetic material for assessing population parameters in this species using molecular markers. One of the major contributions of molecular ecology to conservation has been the evaluation of the viability of populations by detecting the partitioning of genetic diversity within and among populations (Haig 1998). An assessment of population structure with molecular markers in *L. acutus* is the best way to evaluate and provide a more comprehensive picture of its population biology, detect fragmented and vulnerable populations, and therefore allocate resources for its conservation. (e.g., Cassens et al. 2005; Haig 1998; Castello 1996).

This chapter will also test the hypothesis of non differentiation between populations of *L. acutus* between the eastern and western North Atlantic. The extent of genetic differentiation among regional groups of *L. acutus* and the genetic flow among populations will be evaluated at the mitochondrial and nuclear genome levels.

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3. Population size and main threats in L. acutus

According to Waring et al. (2006, 2007) the total number of *L. acutus* along the western North Atlantic is unknown. The available estimates are scarce and have been carried out in only a few places and for a few months (Waring et al. 2007; Kingsley and Reeves 1998). The best abundance estimate for this species in the western North Atlantic [51640 animals (CV=0.38) with a minimum population of 37904 dolphins] was reported by Waring et al. (2007) based on an aerial survey from July-August 1999. No population estimate exists for the species in the eastern North Atlantic. A shipboard survey of the North Sea and adjacent waters in July 1994 estimated 11760 (5867-18528) dolphins of *Lagenorhynchus spp.*, most of which are likely to have been white-beaked dolphins (*L. albirostris*; Hammond et al. 2002).

It is well known that *L. acutus* inhabits one of the most intensively fished waters in the world, especially over the continental shelf (Morizur et al. 1999). Mass strandings (possibly caused by interaction with pelagic trawling), high by-catch rate in fishing nets, and commercial hunting have been reported as the major threats affecting these populations (Waring et al. 2006; Reeves et al. 1999; Reeves and Leatherwood 1994; Alling and Whitehead 1987). In addition, it is known this species has been subjected to direct exploitation in the Faeroe Islands (where more than 500 were killed in the drive fisheries in one year) and Southwest Greenland, where the annual catch has been estimated as approximately 50 individuals (Reeves et al. 1999; Reeves and Leatherwood 1994). These historical harvests may be a threat to the population; however no accurate population size estimates are available for the North Atlantic. Furthermore, little is known about their conservation status. In particular, little is known about their effective population size, life history and stock boundaries, all of which are essential for effective management.

The effect of the foregoing factors on the survival of species can be studied using DNA markers (see Chapter I). If the effective sizes in *L. acutus* populations have been reduced recently by human impact or changes in habitats, low genetic variability at both nuclear and mitochondrial markers and a deviation from the equilibrium expected in stable, well-

preserved populations are to be expected. This hypothesis will be evaluated in this chapter using the bottleneck program (Cornuet and Luikart 1996) which test a deviation from the mutation-drift equilibrium using different statistics and several mutation models (see next section) and with the M-Garza statistic (Garza and Williamson 2001) which tests recent population reduction using the mean ratio of number of alleles to the range in allele size in microsatellite data.

MATERIALS AND METHODS

1. Sample Collection

A total of 192 samples of *L. acutus* were collected from throughout its known geographic range. Samples from the western North Atlantic belong to two temporally unrelated populations: ancient teeth samples belong to one possible stranding event in Barnstable, Cape Cod, Massachusetts, in the 19th century, and modern teeth samples from different locations, mainly the Gulf of Maine, Cobscook Bay, Lingley cove. Tissue and teeth samples from the eastern North Atlantic belong to different geographic locations in Scotland, the English coast, the Netherlands and Ireland (Table III.1; Figures III.2a and III.2b).

Bone and teeth samples were collected from different museums in Holland: the Museum of Natural History of Rotterdam and the Museum of Natural History in Leiden, and the Smithsonian Museum in the United States. Samples from living and stranded dolphins were collected through cooperative agreements with Stranding Networks from Scotland (Dr. Bob Reid and Mary Harmen), England (Dr. Rob Deville) and with the Marine Institute in Bergen, Norway (Dr. Arne Bjorne). Scientific collaboration was also received from Dr. Emer Rogan and Dr. Luca Mirimin from the Marine Research Institute in Ireland.

2. DNA extraction

Total genomic DNA was extracted from tissue following the procedure described in chapter II. Bone and teeth samples were cleaned with a 10% solution of bleach for 2 hours; and after rinsing with water overnight, they were placed under ultraviolet light for 20 minutes prior to extraction. The samples were extracted in a laminar flow hood, cleaned before and after each extraction with a 10% solution of bleach and 100% ethanol to prevent contamination.

Table III.1. Samples obtained from *Lagenorhynchus acutus* in each region.

COUNTRY	COUNTY	N	LOCATION	n
SCOTLAND		17	North Kessock	1
			East Suisnish, Raasay	1
			Kyle Of Durness	1
			Mallaig	1
			West Beach, Lossiemouth	1
			Ardersier	1
			S. Kessock, Inverness	1
			Kiltearn, Evanton	1
			Balnaglack, Ardersier	1
			Brora	7
			Near Evanton	1
	ORKNEY	7	Scapa Beach	2
			Ayre Sound, Sanday	1
			South Burray	3
			Newark, Deerness	1
	SHETLAND	16	Grunna Voe	1
			Braewick Eshaness	1
			Whiteness Voe	1
			Sand	1
			Catfirth Voe	1
			Otters Wick, Yell	1
			Baltasound, Unst	1
			Maryfield, Bressay	2
			Uyea Sound, Unst	1
			Weisdale Voe, Mainland	6
	STRATHCLYDE	3	Glen Forsa Bay, Mull	1
			Loch Beg, Isle Of Mull	1
			Loch Crinan	1
	WESTERN ISLES	12	Eochar	1
			Sollas, North Uist	1
			Borve Beach, Harris	1
			Ormiclate, South Uist	1
			Knockintorran, N. Uist	1
			Stornoway Harbour	1
			Usinish, South Uist	1
			Knockline, N. Uist	1
			Culla Bay, Benbecula	1
			Kilpheder, South Uist	1
			Rubha Ardvule, S. Uist	1
			Bornish, South Uist	1

Table III.1. Continuation. Samples obtained from *Lagenorhynchus acutus* in each region.

ENGLAND				
	Northumberland	7	Beadnell	1
	Devon		Saunton Sands	1
	Cleveland		Redcar	1
	West Glamorgan		Rhossili Beach	1
	Humberside		Bridlington	1
	Cornwall		Polzeath Beach	1
	North Yorkshire		Scarborough	1
Subtotal				62
IRELAND		46		46
THE NETHERLANDS		24		24
Subtotal eastern North Atlantic		122		122
UNITED STATES COASTS		70		
	Gulf of Maine			39
	Massachusetts			11
	Virginia			2
	North Carolina			3
	Unknown origin			9
	Offshore samples			6
Subtotal western North Atlantic				70
TOTAL				192
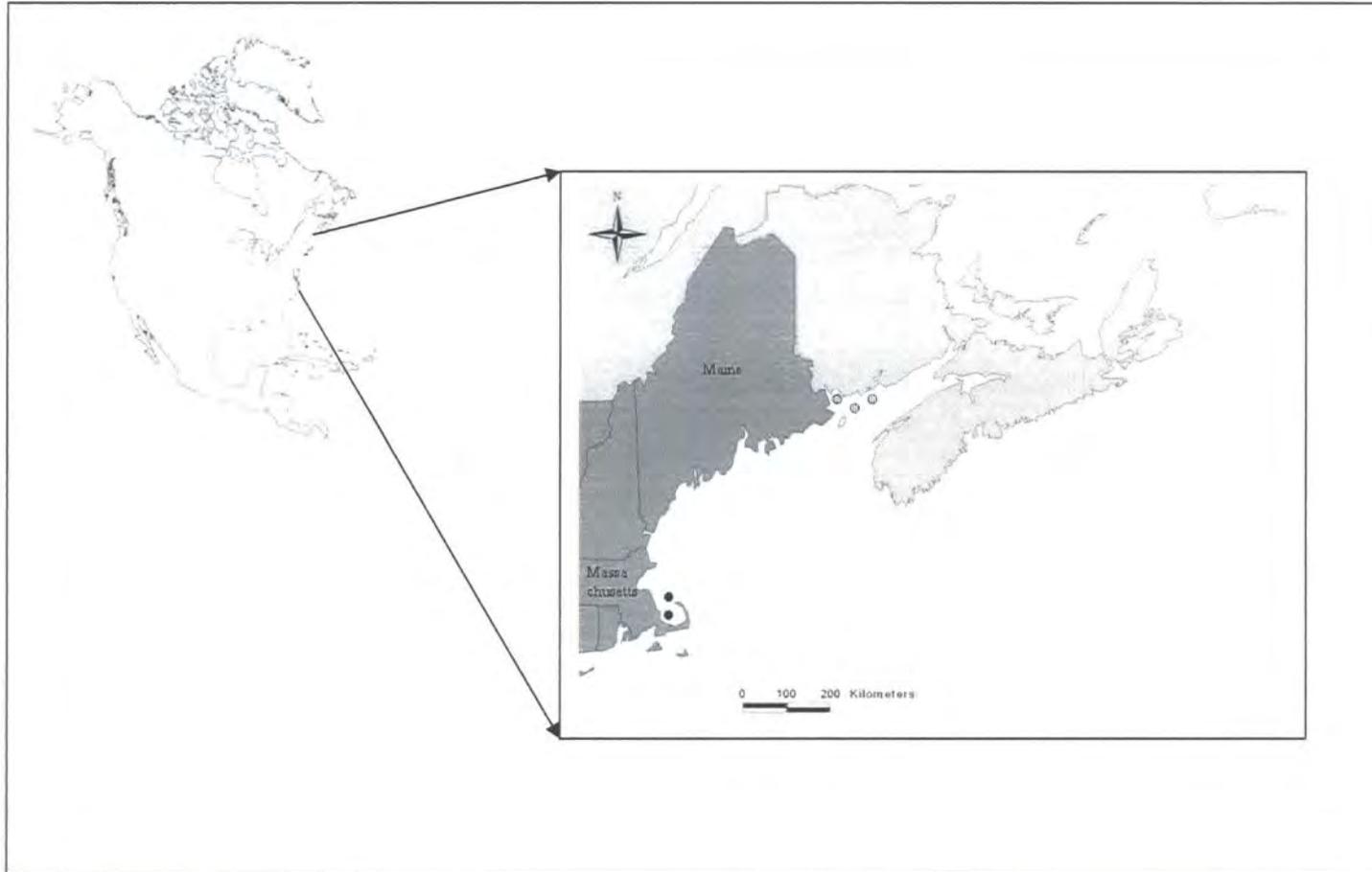
Figure III.2a. Sampling localities for *L. acutus* in the western North Atlantic.*

*Black circles represent samples from the western Ancient population and grey dashed circles represent samples from the western Modern population.
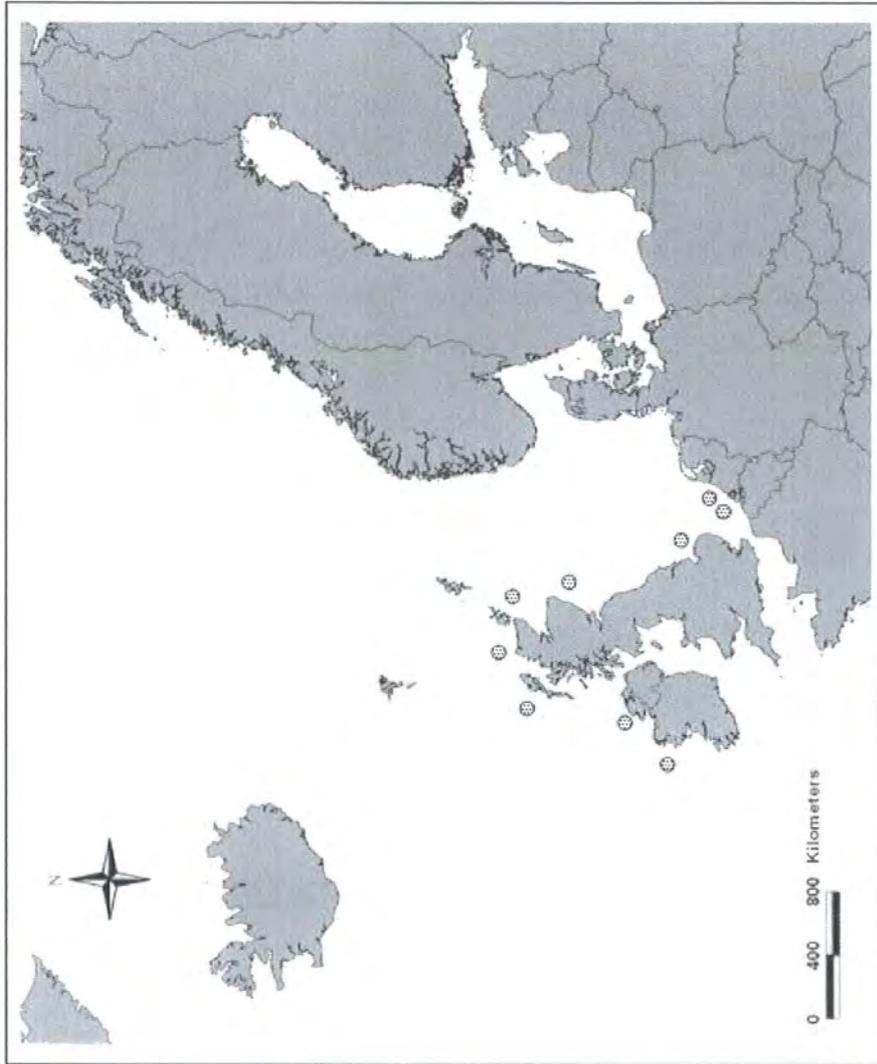
Figure III.2b. Sampling localities for *L. acutus* in the eastern North Atlantic.

The teeth or bones were ground using a pestle and mortar, paying special attention to decontamination and sterilization by autoclave before a new sample was extracted. They were then digested in 2 ml lysis solution for 48 h at 37°C (0.5M EDTA, pH 8.0, 0.1M Tris-HCl and 0.5% SDS). After digestion the DNA was extracted using a Qiagen PCR purification kit, following the protocol recommended for cleaning PCR products, the samples were diluted in 0.1mM Tris-EDTA buffer and placed at -20°C until use. All extractions were conducted with disposable equipment and extraction controls to detect and reduce any sample contamination.

3. Mitochondrial control region (mtDNA) amplification and sequencing

Three fragments of the maternally inherited mtDNA control region were amplified for a subset of samples under the following conditions: 20-50 ng of DNA, 10x PCR buffer, 1.5 mM $MgCl_2$, 50-100 ng of primers, 2.5 mM dNTP and 1U of Taq polymerase (except for bone samples, where 2U was used). Amplifications were conducted in a MJ Research thermocycler with the following cycle conditions: 94°C 2 min. followed by 35 cycles of 94°C 30 sec (46 cycles in teeth and bone samples), 54°C 30 sec and 72°C 30 sec. A 600 bp fragment was amplified using universal primers MTCRF (5´- TTC CCC GGT CTT GTA AAC C- 3´) and MTCR-R (5'-ATT TTC AGT GTC TTG CTT T-3') from Hoelzel and Green (1998). This 600 bp fragment did not amplify in bone and teeth samples; thus internal primers (flanking internal regions in the 600 bp fragment) were used to amplify a smaller fragment (280-317 bp) (AcuF: 5' TGT ACA TGC TAT GTA TTA T 3' AcuR: GCT TTA ACT TAT CGT ATG G- 3'). After amplification the samples were purified using Qiagen columns (Qiagen, Inc.) and were directly sequenced in an ABI 377 automated sequencer. The sequences were aligned using the Clustal X programme (1.83) from Thompson et al. (1997) and edited using the programme Chromas Pro (www.technelysium.co.au).

3.1. mtDNA analysis

Samples were initially divided into four geographic zones in the eastern North Atlantic: Scotland (N=34), the Netherlands (N=35), Ireland (N=28) and England (N=10) and in two

different populations in the western North Atlantic: referred to hereafter as "western Ancient" (N=26) and "western Modern" (N=29). After population structure analyses (see results), samples from Scotland, England and Ireland were pooled together in a UK group.

The extent of genetic variation in the control region was assessed by examining both haplotype (h) and nucleotide diversity (π), using Arlequin v 3.11 (Excoffier et al. 2007) and DNAsp v 4.0 (Rozas et al. 2003). Genetic diversity, also called haplotypic diversity, is defined as the probability that two randomly chosen haplotypes are different in the sample. It is calculated following Nei (1987) as:

$$H: n/n-1 \left(1-\sum_{i=1}^{k} p_i^2\right)$$

Where k is the number of haplotypes in the sample, n is the number of gene copies, and p_i is the frequency of the ith haplotype. Nucleotide diversity (π) or average gene diversity over L loci is defined as the probability that two randomly chosen homologous nucleotide sites are different. It is given by

$$\pi_n = \frac{\sum_{i=1}^{k} \sum_{j<i} p_i \, p_j \, \hat{d}_{ij}}{L}$$

Where n is the sample size, k is the number of haplotypes, p_i and pj are the frequencies of each haplotype (i and j), dij is the estimate of the number of mutations that have taken place since the divergence of haplotypes i and j and L is the number of loci (Tajima 1993).

The degree of genetic differentiation among geographic samples was determined using the Analysis of Molecular Variance (AMOVA) with the modifications proposed by Excoffier et al. (1992) and implemented in Arlequin v 3.11 (Excoffier et al. 2007). This analysis is based on analyses of variance of gene frequencies, and it takes into account the number of mutations among molecular haplotypes (Excoffier et al. 2007). The variance components of

gene frequencies were partitioned between geographic regions (groups): western North Atlantic and eastern North Atlantic; among populations (UK, The Netherlands, western Ancient and western Modern) and within groups and within populations. The differentiation was quantified using the fixation index, Fst (Wright 1951; Excoffier et al. 1992) and the statistical significance of the variance components and fixation statistics were tested using a non-parametric permutation approach with 10000 permutations.

The phylogenetic relationships among haplotypes were examined generating a neighbour-joining tree for the complete set of mtDNA haplotypes using the Tamura and Nei substitution model (Tamura and Nei 1993), and the analyses were conducted using MEGA version 4.0 (Tamura et al. 2007) and PAUP* v. 4.0b10 (Swofford 2002). In addition a median-joining network tree was built to infer the ancestral relationships among haplotypes using the programme Network version 4.5.0.0 (Bandelt et al. 1999).

The distribution of the number of observed differences between pairs of DNA sequences (mismatch distribution) (Rogers & Harpending 1992) was used as an assessment of demographic history of the population, using the program Arlequin v 3.11 (Excoffier et al. 2007). Sudden demographic expansion usually leads to unimodal shapes in the distributions of pairwise differences, while multimodal shapes are frequently associated with populations that have been stable over long periods of time (Rogers and Harpending 1992).

The parameters of the demographic expansion τ, θ_0, and θ_1 were calculated using the method suggested by Schneider and Excoffier (1999). The confidence intervals for these parameters are obtained by a parametric bootstrap approach that assumes that the data are distributed according to the sudden expansion model. The calculations were carried out following the principles explained by Schneider and Excoffier (1999) and using a coalescent algorithm modified from Hudson (1990). The hypothesis that the observed data fit the sudden expansion model was tested using the sum of square deviations (SSD) (Schneider and Excoffier 1999) and the raggedness index (Harpending 1994). These analyses test "the validity of a stepwise expansion model based on the sum of squares deviations between the observed and expected mismatch, with probability values (P); non-

significant mismatch values and non-significant raggedness values suggest population expansion" (Elmer et al. 2007 p. 7).

The coalescence time of expansion in years (t) was calculated using the relationship $\tau=2\upsilon t$, where τ represents the mode of the mismatch distribution (in units of evolutionary time) and υ is the mutation rate for the sequence used. The υ value was calculated as suggested by Rogers and Harpending (1992), using the formula $\upsilon=\mu k$, where μ is the mutation rate per nucleotide and k is the number of nucleotides evaluated.

Demographic parameters were estimated using three different evolutionary rates: (i) The value calculated by Hayano et al. 2004 (λ=2.11-2.99 × 10^{-8}) for the hypervariable region of the control region (~500 bp) for *L. obscurus* and *L. obliquidens*. (ii) The estimate by Harlin et al. 2003 (λ=6.3-7.0 × 10^{-8}) based on comparisons between *Phocoena phocoena* and *L. obscurus* for the same region and (iii) A recent estimate of mutation rate for the control region of μ=5 × 10^{-7} by Ho et al. (2007). The mutation rates per site per year (μ) from λ values were calculated using a generation time of 10 year suggested by Ferrero and Walker (1996).

Population growth frequently has an effect on the distribution of several test statistics of neutrality (Sano and Tachida 2005); thus in this study Fu's test (Fu 1997) and Tajima's test (Tajima 1989b) were used to evaluate the demographic history of *L. acutus* populations. Fu's test (Fu 1997) which is based in the infinite-site model without recombination and It is especially useful to test for an excess of rare alleles in short DNA sequences. This statistic is very sensitive to population demographic expansion, which leads to large negative F values (Fu 1997). In this test a P value of 0.02 is considered significant (Fu 1997).

Tajima's D-statistic (Tajima 1989b) also assumes an infinite-site mutation model (each mutation will occur in a new position), and it compares two measures of divergence based on the number of segregating sites (θ) and the average number of nucleotide differences (π) to test whether the region is neutral or under selection and whether or not the population is in equilibrium (Tajima 1989a; Tajima 1989b). Positive values of Tajima's D ($\theta<\pi$) are

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indicative of either balancing selection or admixture of two genetically different populations (Pichler 2002); whereas negative values suggest a selective sweep or sudden expansion. Significant D values are often related to factors other than selection such as population bottlenecks and subsequent expansion or heterogeneity of mutation rates (Aris-Brosou and Excoffier 1996; Excoffier et al. 2007). The significance of Tajima's D was determined by generating 1000 random samples under the assumption of selective neutrality with a coalescent simulation algorithm (Hudson 1990). Both tests were evaluated using Arlequin v 3.11 (Excoffier et al. 2007) and DNAsp (Rozas et al. 2003).

A Bayesian sampling coalescent approach implemented in the IM (Hey and Nielsen 2004) and Mdiv (Nielsen and Wakeley 2001) programmes was used to evaluate whether or not the observed genetic pattern in the populations of *L. acutus* studied fit to an isolation-with-migration model under the finite site mutation model (HKY), which takes into account the possibility of multiple mutations at each nucleotide site, transition/transversion bias and differences in nucleotide frequencies (Hasegawa et al. 1985).

The demographic model considered by Nielsen and Wakeley (2001) and Hey and Nielsen (2004) evaluates several population parameters under the assumption that two populations diverged from a single common ancestor in a generation time (t) in the past. These parameters are the population size of the ancestral population (θ) = 2 × effective population size × mutation rate ($\theta=2Ne\mu$); the migration rate between populations 1 and 2 (M) = 2 × effective population size × migration rate (M=2Nef×m), and the time in which the two populations split in the past (T) = divergence time/ 2 × effective population size (T=t/2Nef).

The IM programme was run with the following parameters: burn-in 100000 –q1 120 (q1= scalar for θ) –m1 50 –m2 50 (m=migration) –t 300 (time) –u 10 (generation in years); the programme was run three times with different random number seeds. The Mdiv programme was run twice using 5×10^6 and 10×10^6 chains and a burn-in of 10% (500000 and 1000000 respectively) as recommended by the author.

Current genetic flow among populations and population sizes were estimated using Migrate version 2.0 (Beerli 1997-2004; Beerli and Felsenstein 1999), however the estimates obtained under the assumption of this program were unreasonably high, therefore Fst statistics were used as an indirect measure of genetic flow, and current population sizes were calculated using θ_π. Both parameters were obtained as indicated in Arlequin version 3.11 (Excoffier et al. 2007).

4. Microsatellite loci

In order to determine genetic variability and differentiation for biparental gene flow among geographic samples, twenty cetacean-specific loci obtained from published reports were evaluated (Table III.2). The PCR reactions were performed in the presence of 20-50 ng of DNA (10 μl for teeth samples) for a final volume of 20 μl. The reaction mix contained 200 nM of each primer (the forward primer was labelled using fluorescence to allow detection by the program sequencer), 0.5-0.75 mM MgCl2, 0.1-0.36 mM dNTPs and 0.2 U Taq polymerase (BiolineTM). The annealing temperature and cycle recommendations from each reference are given in Table III.2.

4.1. Microsatellite analyses

The PCR conditions for DO8, D22, GT136, FCB4, IgF1, Kwm2a and texvet5 were: Denaturation at 95°C for 5 min, 35 cycles at 94°C for 45 sec, 1 min 30 sec at locus-specific annealing temperature, extension at 72°C for 1 min 30 sec. PCR conditions for Ev37 and Ev94 (see Valsechi and Amos 1996). PCR conditions for Textvet 7: Denaturation at 95°C for 5 min 35 cycles at 94°C for 40 sec, 1 min 30 sec at locus-specific annealing temperature and 1 min 40 sec at 72°C. PCR conditions for Gt011: Denaturation at 95°C for 3 min, 35 cycles at 94°C for 1 min, 1 min 30s at locus-specific annealing temperature and 10 sec at 72°C.

Table III.2. Microsatellite DNA loci standardized in *Lagenorhynchus acutus*.

	Locus	Annealing temperature (°C)	Allele sizes	References
1	415/416	47	229-233	Amos et al. 1993
2	*D08	54	103-109	Shinohara et al. 1997
3	*D22	49	114-122	Shinohara et al. 1997
4	D28	52	145-149	Shinohara et al. 1997
5	*EV37	48/57	203-211	Valsechi and Amos 1996
6	*FCB4	55	213-231	Buchanan et al. 1996
7	FCB5	55	140-144	Buchanan et al. 1996
8	*GT011	59	104-112	Bérubé et al. 1998
9	GT101	43	124-130	Andersen et al. 2001
10	*GT136	52	115-127	Andersen et al. 2001
11	**Igf1	59 (57)	138-152	Andersen et al. 1997
12	KWM12a	54	164-168	Hoelzel et al. 1998b
13	**KWM2a	49 (47)	131-154	Hoelzel et al. 1998b
14	KWM1b	50	186-190	Hoelzel et al. 1998b
15	MK8	56	Monomorphic	Krutzen et al. 2001
16	TAA031	55	Monomorphic	Palsbøll et al. 1997
17	**Textvet5	52 (54)	182-188	Rooney et al. 1999
18	**Textvet7	59 (57)	177-195	Rooney et al. 1999
19	EV5	48/56	Monomorphic	Valsechi and Amos 1996
20	**EV94	48/56 (46/56)	268-294	Valsechi and Amos 1996

*Polymorphic microsatellites with more than four alleles that were selected.
**Primers evaluated with bone and teeth samples, annealing temperatures are in parentheses.

After amplification, microsatellite loci were run on polyacrylamide gels using an 377 ABI automated sequencer and analyzed using ABI GenescanTM and Genotyper TM . The first program permits sizing the alleles in the PCR product using a labelled DNA-size ladder (ROX). The alleles were then scored with the second program, and the peaks were considered reliable when the amplitude was higher than 100. The majority of loci showed a clear pattern of one prominent peak that was scored as the true allele; few of them showed presence of stutter peaks.

To identify and correct genotyping errors (i.e. to check evidence for scoring error due to stuttering, large allele dropout or evidence for null alleles), the program Microchecker (Van Oosterhout et al. 2004) was used.

Four populations were evaluated with microsatellite loci: Scotland, Ireland, The Netherlands and western Modern. Microsatellite variation was examined by estimating the number of alleles per locus, gene diversity and allelic richness using the programme Fstat vers. 2.9.3 (Goudet 2001). Regional differences in frequencies and deviation from the Hardy-Weinberg equilibrium were tested using the GENEPOP 1.2 programme (Raymond and Rousset 1995) and Arlequin v 3.11 (Excoffier et al. 2007). Linkage disequilibrium to test the null hypothesis of independence between genotypes was tested using Fstat vers. 2.9.3 (Goudet 2001).

The heterozygote deficiency test and the heterozygote excess test (Rousset and Raymond 1995) were calculated and subject to sequential Bonferroni correction (Rice 1989). A Markov chain estimate of Fisher's exact test was also used in order to test the null hypothesis that allelic distribution was identical across populations (Guo and Thompson 1992). Population differentiation was assessed using the fixation index (Fst) approach of Weir and Cockerham (1984) and Rst (Slatkin 1995).

To test the hypothesis that the populations are in mutation-drift equilibrium, the sign test in the Bottleneck programme (Cornuet and Luikart 1996) was used. This test evaluates the differences between observed and expected heterozygosities across all loci in a population

sample. In a population that underwent a bottleneck, a transient excess of heterozygosity is expected; thus a higher-than-expected observed heterozygosity would be found (Cornuet and Luikart 1996). This test works under three different mutation models proposed for microsatellite data: the Infinite Allele Model (IAM, Kimura and Crow 1964) the Stepwise Mutation Model (SMM, Kimura and Ohta 1978) and the Two Phase Model (TPM, Di Rienzo et al. 1994). The SMM model assumes that mutation in microsatellite alleles arises by the loss or gain of a single tandem repeat; the IAM model assumes that a mutation involves any number of tandem repeats; and under the TPM model, a mutation involves a gain or loss of X repeats. The TPM model is considered intermediate to the SMM and IAM models.

The reduction in population size was also tested with the statistic M, proposed by Garza and Williamson (2001). This method uses microsatellite data under the SMM model and analyzes two characteristics of the tandem repeat units to examine the statistic M. This is based on the quantitative diversity (frequency of alleles and total number of alleles, k) and the spatial diversity (the distance between alleles in number of repeats and the overall range in allele size, r), so that M=k/r. In recently reduced populations M is expected to be smaller than in an equilibrium population (Garza and Williamson 2001).

RESULTS

1. Genetic variation at the mtDNA control region

A total of 162 sequences from *L. acutus* were obtained. A fragment of 317 bp was analyzed in samples from the eastern North Atlantic, whereas samples from the western North Atlantic were analyzed using 280 bp. Eastern and western North Atlantic samples were compared using the shortest fragment.

- ### *Eastern North Atlantic*

Among the four geographic regions in the eastern North Atlantic, 34 haplotypes and 27 polymorphic sites were found (Figure III.3). Four shared haplotypes were identified among regions (Scotland, England, Ireland and The Netherlands), and unique haplotypes were found in all populations. Over all samples, haplotypic (gene) diversity (Hd: 0.934 ± 0.01) was similar to values found in other odontocete populations in which the values ranged from 0.7 to 0.92 (See Pichler and Baker 2000). However, low nucleotide diversity values were found (π: 0.0098 ± 0.0057) similar to those in species that have undergone a population bottleneck (see review in Luikart and Cornuet 1998). Table III.3 shows the diversity values for each region and for the UK population (Scotland, England and Ireland). In the eastern North Atlantic the F_{st} and ϕ_{st} values showed a small, but significant differentiation between Scotland and The Netherlands (F_{st}: 0.0614 P<0.0090) and The Netherlands and Ireland (F_{st}: 0.0597 P<0.0180) (Table III.4).

- ### *Western North Atlantic*

A total of 18 haplotypes and 17 polymorphic sites were identified in western Modern samples (N=29), and 13 haplotypes were defined by 13 polymorphic sites in the western Ancient samples (N=26) (Figure III.4). Seven shared haplotypes were found between the two populations. Haplotypic diversity and nucleotide diversity values for both populations are shown in Table III.3.

```
                     1 6 6 6 8 9 9 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2     A      B      C      D
*Haplotypes          1 2 5 9 2 4 0 2 2 2 4 6 6 6 7 8 9 5 6 7 7 7 7 8 9 9    N=35   N=10   N=28   N=34
                           9 4 7 8 8 2 5 7 0 7 9 9 3 4 6 7 8 2 5 7

Hap 1    A T T T T C C G T T T A T G A C A T A C T T T T T T G               1      2      3      2
Hap 2    . . . . . . . . . . . . . . . . . . . . . . . A . . .               1
Hap 3    . . C . . . . A . . . . . . . . . . . . C . T . . . .               3      2      5      3
Hap 4    . . . . . . . A . . . . . . . . . . . . . . T . . . .               1             1      3
Hap 5    . C . . . . . . . . . . . . . . . . . . . . T . . . .               2      1
Hap 6    . C . . . . . . . . . . . . . . . . . . . . T . . . A               1
Hap 7    . . . . . . . . . . . . . . . . . . . . . . T . . C .               4      1      2      3
Hap 8    . . . . . . . . . . . . . . . . . . . . . . T . . . .               2      2      5      2
Hap 9    . . . . . . T . . . . G . . G . . . . . . . T . . . .               1
Hap 10   . . . . . . T . . . . . . . . . . . . . . . T . . . .              13      1             2
Hap 11   . . . . . . . . . . . . . . . . . . . . C . C . . . .               2
Hap 12   . . . . . . . . . . . . . . . . . . . . . . C . . . .               1
Hap 13   . . . . . . . A . . . . . C . . . . . . . . C . . . .               2             2      4
Hap 14   . . . . . . . A . . . . . . . . . . . . . . C . . . .               1
Hap 15   . . . . . T . . . . . . . C . . . . . . . . . . . . .                      1
Hap 16   . . . . . . . . . . . . . . . . . . . G . . . . . . .                             1      1
```

Figure III.3. Haplotypes and nucleotide positions in the eastern North Atlantic.

*Haplotypes were defined using a fragment of 317 bp. A: The Netherlands, B: England, C: Irish Sea, D: Scotland.

*Haplotypes	1	6	6	6	8	9	9	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	A N=35	B N=10	C N=28	D N=34
	1	2	5	9	2	4	0	2	2	2	4	6	6	6	7	8	9	5	6	7	7	7	7	8	9	9						
							9	4	7	8	8	2	5	7	0	7	9	9	3	4	6	7	8	2	5	7						
Hap 17	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	.			1	
Hap 18	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.			1	
Hap 19	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.			1	
Hap 20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.			1	
Hap 21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.			1	
Hap 22	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.			1	
Hap 23	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.			1	
Hap 24	.	.	.	.	.	.	.	A	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.			2	
Hap 25	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A				4
Hap 26	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				1
Hap 27	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				2
Hap 28	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.				1
Hap 29	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.				1
Hap 30	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.				1
Hap 31	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				1
Hap 32	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.				1
Hap 33	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.				1
Hap 34	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.				1

Figure III.3. Continuation. Haplotypes and nucleotide positions in the eastern North Atlantic.

A: The Netherlands, B: England, C: Irish Sea, D: Scotland.

Table III.3. Diversity estimates in the eastern and western North Atlantic.*

Region	N	H	P	Hd	π	K
The Netherlands	35	14	15	0.8471 ± 0.0528	0.0089 ± 0.0054	2.622
England	10	7	10	0.9333 ± 0.0620	0.0086 ± 0.0056	2.533
Ireland	28	15	17	0.9312 ± 0.0274	0.0101 ± 0.0060	3.079
Scotland	34	18	16	0.9554 ± 0.0156	0.0102 ± 0.0060	3.073
UK (England, Ireland and Scotland)	72	28	22	0.9413 ± 0.0118	0.0097 ± 0.0056	3.005
Overall (eastern NA)	107	34	28	0.9340 ± 0.010	0.0098 ± 0.0057	2.949
Western Modern	29	18	17	0.9261 ± 0.0345	0.0096 ± 0.0054	2.638
Western Ancient	26	13	13	0.8923 ± 0.0475	0.0119 ± 0.0070	3.240
Over all samples	162	43	30	**0.9170 ± 0.0120	**0.0095 ± 0.0005	2.766

*N= number of samples by region. H: Number of haplotypes; P: Polymorphic sites; Hd: Haplotypic diversity; π: nucleotide diversity; K= Average number of nucleotide differences. **Over all samples the genetic diversity and nucleotide diversity values were computed excluding samples from western Ancient origin.

Table III.4. Fst and φ_{st} values in eastern populations.*

Region	The Netherlands	England	Ireland	Scotland
The Netherlands		0.0274	**0.0727**	**0.0476**
England	0.0006		-0.0358	-0.0011
Ireland	**0.0597**	-0.0339		0.0054
Scotland	**0.0614**	0.0064	0.00677	

*Fst values (below diagonal) and φ_{st} values (above diagonal) were calculated using 10000 permutations. Values in bold are significant at level 0.05.

Figure III.4. Haplotypes and nucleotide positions between eastern and western North Atlantic.

*Haplotypes	15	38	41	62	65	64	79	82	94	90	101	109	120	124	127	128	129	161	162	165	170	187	199	246	259	263	274	276	277	278	WM N=29	WA N=26	UK N=72	TN N=35
Hap 1	A	A	T	T	T	T	C	T	C	C	A	G	G	T	T	T	C	T	T	G	C	A	T	T	A	C	T	T	T	T	7	2	8	1
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	1			
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	3		2	
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	1			
Hap 5	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	1	2	6	2
Hap 6	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1		
Hap 7	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	1	3	3	14
Hap 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	.	1			
Hap 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	1		1	
Hap 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	4	2	16	6
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	C	.	1			
Hap 12	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	1			
Hap 13	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	1	1	1	
Hap 14	.	.	C	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	1	8	10	3
Hap 15	.	.	C	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	C	.	.	1			
Hap 16	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	1			
Hap 17	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	1			
Hap 18	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	1			
Hap 19	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.			1	
Hap 20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	T	.	.	.	.			1	
Hap 21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.			1	
Hap 22	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.		1	4	1
Hap 23	.	.	C	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.			1	

*Haplotypes were defined using a fragment of 280 bp. WM: western modern, WA: western ancient, UK: Samples from UK, Scotland and Ireland, TN: The Netherlands.

Figure III.4. Continuation. Haplotypes and nucleotide positions between eastern and western North Atlantic.

*Haplotypes were defined using a fragment of 280 bp. WM: western modern, WA: western ancient, UK: Samples from UK, Scotland and Ireland, TN: The Netherlands.

	15	38	41	62	65	64	79	82	94	90	101	109	120	124	127	128	129	161	162	165	170	187	199	246	259	263	274	276	277	278	WM N=29	WA N=26	UK N=72	TN N=35
	A	A	T	T	T	T	C	T	C	C	A	G	G	T	T	T	C	T	T	G	C	A	T	T	A	C	T	T	T	T				
Hap 24	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	C	.			2	
Hap 25	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.			1	3
Hap 26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A				1
Hap 27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C				2
Hap 28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.				1
Hap 29	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.				1
Hap 30	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			5	
Hap 31	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.			1	
Hap 32	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			2	
Hap 33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.			1	
Hap 34	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.			1	
Hap 35	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			1	
Hap 36	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.			1	
Hap 37	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.			1	
Hap 38	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.			1	
Hap 39	.	G	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.		1		
Hap 40	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	T	.	.	.		1		
Hap 41	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.		2		
Hap 42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		1		
Hap 43	.	.	G	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		1		

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- *Western vs eastern North Atlantic*

Between the two regions 43 haplotypes were found defined by 30 polymorphic sites, 17 of which were parsimony informative (Figure III.4). Values for haplotypic and nucleotide diversity over all samples -excluding the western Ancient population- are shown in Table III.3.

1.1. Differentiation among populations

An AMOVA was performed to test differentiation among groups, dividing the samples into two groups: eastern (The Netherlands plus UK) and western North Atlantic (western Ancient plus western Modern). The results indicate that only 7.27% of the variance in haplotype frequencies could be explained by the differences between western and eastern populations, while 96.31% of the variance could be explained as a result of the differences within populations in each geographic region (Table III.5).

The Fst values suggest that there is no differentiation between the western North Atlantic and the eastern North Atlantic as suggested in previous studies by Mikkelsen and Lund (1994). In fact the western Modern population was not differentiated from the UK population (φst: -0.0005 P<0.4118); However, when individuals from the southern North Sea were evaluated (The Netherlands), some degree of differentiation was found between each population either side of the North Atlantic (Table III.6).

1.2. Phylogenetic relationships among haplotypes

No clear association among haplotypes from the different regions was found, either in the neighbour joining tree or in the median-joining network (Figure III.5a); however the star-like shape in parts of the network tree suggests one or more sudden expansions of *L. acutus* in the North Atlantic (Figure III.5b).

Table III.5. AMOVA performed to evaluate the differentiation among regional samples of *L. acutus*.

Source of variation	df	Variance components	Variance explained (%)	P	Fixation indices
Among groups	1	-0.05076	-3.58	0.6738	φ_{ct}: -0.03583
Among populations within groups	2	0.10308	7.27	0.00030	φ_{sc}: 0.07023
Within populations	158	1.36460	96.31	0.00040	φ_{st}: 0.03692

Table III.6. F_{st} **and** φ_{st} values between populations in the western and eastern North Atlantic.*

Region	UK	The Netherlands	Western Modern	Western Ancient
UK		**0.0667**	0.0126	0.0177
Netherlands	**0.0581**		**0.0887**	**0.0629**
Western Modern	−0.0005	**0.0674**		**0.0438**
Western Ancient	**0.0032**	**0.1144**	**0.0862**	

*Fst values (above diagonal) and φ_{st} values (below diagonal) were calculated using 10000 permutations.
Values in bold are significant at level 0.05.
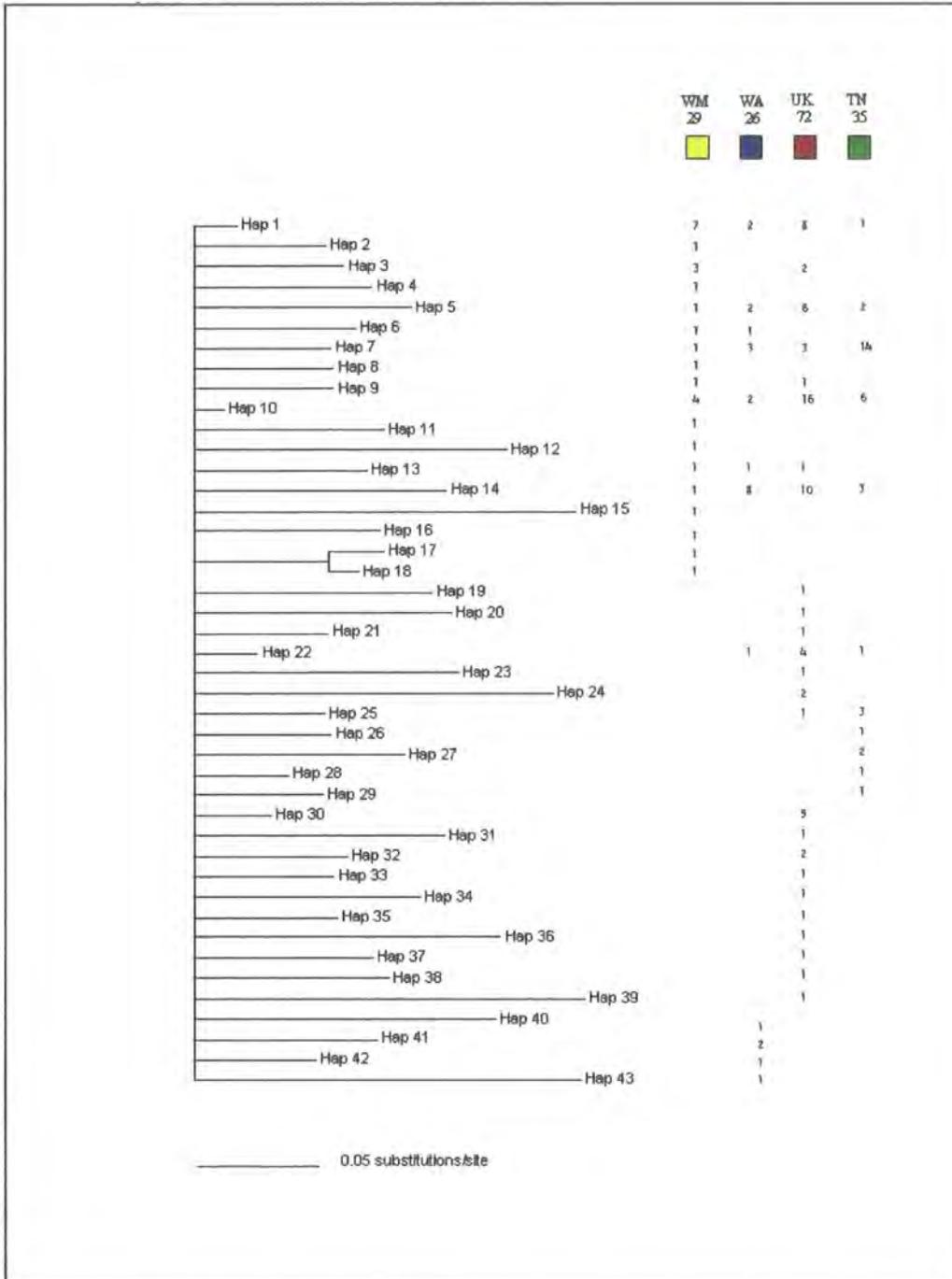
Figure II.5a. Neighbour joining tree showing the relationships among haplotypes in the western and eastern North Atlantic. WM: western Modern, WA: western Ancient, UK: Samples from UK, Scotland and Ireland. TN: The Netherlands.
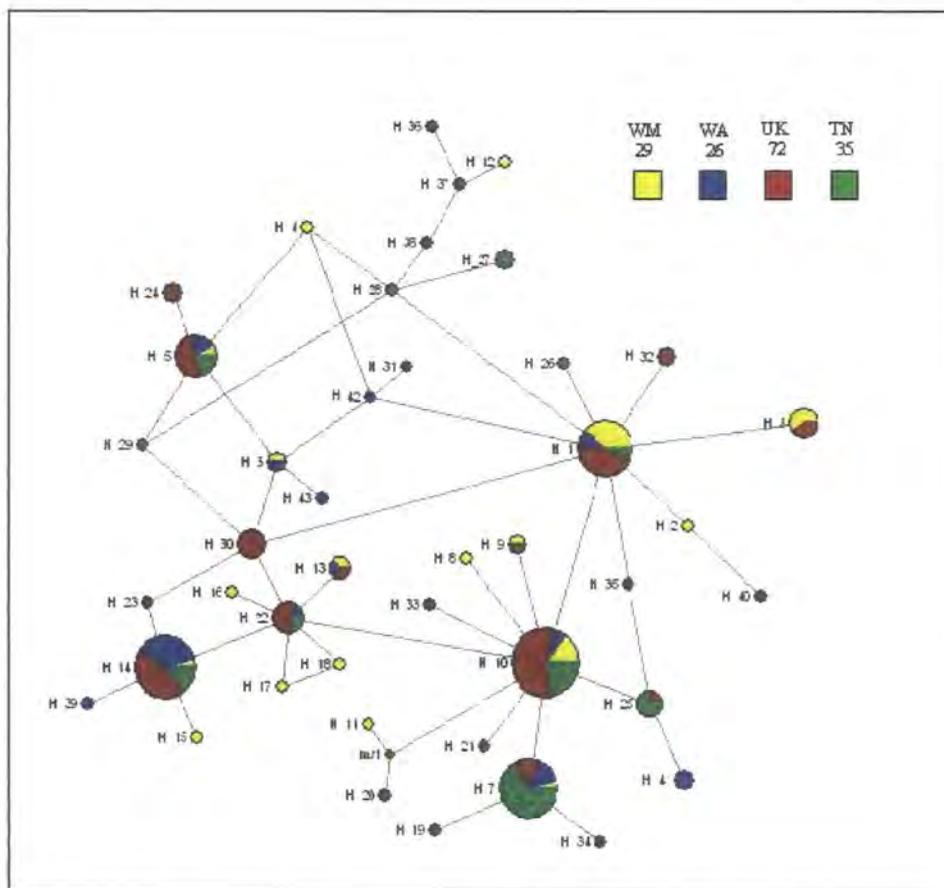
Figure III.5b. Network tree showing the relationships among haplotypes in the western and eastern North Atlantic.

*Line lengths in the network tree are proportional to the number of mutations among haplotypes.

1.3. Mismatch distribution analysis and neutrality tests

The mismatch distribution showed a clear unimodal shape in the UK, western Modern and western Ancient populations; the curve was slightly unimodal in The Netherlands population (Figure III.6). The variance (SSD) and the small, non-significant values of the raggedness index (r) suggested that the curves did not differ significantly from the curves expected under a model of sudden expansion (Table III.7). The sudden expansion in *L. acutus* was also corroborated by the negative, although not significant, values of Tajima's D and the negative and highly significant values of Fu's statistics in all populations, except for the Netherlands (Fs: -3.1684 P> 0.0552) (Table III.7).

The expansion time in *L. acutus* populations, calculated from the parameter τ of the mismatch distribution, ranged from ~14000 to ~7000 years ago using the faster mutation rates (6.3 - 7.0 × 10^{-7} and 5 × 10^{-7}). These values increased by approximately two fold when using the slowest mutation rate (2.11–3.3 × 10^{-7}). The above estimates are consistent with a range expansion after the last glacial maxima in the Pleistocene. The highest value for the effective number of females after the expansion was obtained for the western Modern population, and the lowest value was obtained for The Netherlands population, which was approximately 4 fold smaller than for the other populations (Table III.8).

1.4. Isolation-with-migration model

Two pair of populations were compared using the isolation-with-migration model (UK *vs* The Netherlands and western Ancient *vs.* western Modern). The IM program recovered posterior probabilities only for the migration parameter for western Ancient and western Modern populations. The other parameters did not reach convergence in the several long runs that were performed (more than 120 hours), neither with the IM program nor with the Mdiv program. Low unidirectional past migration was detected from the western Ancient
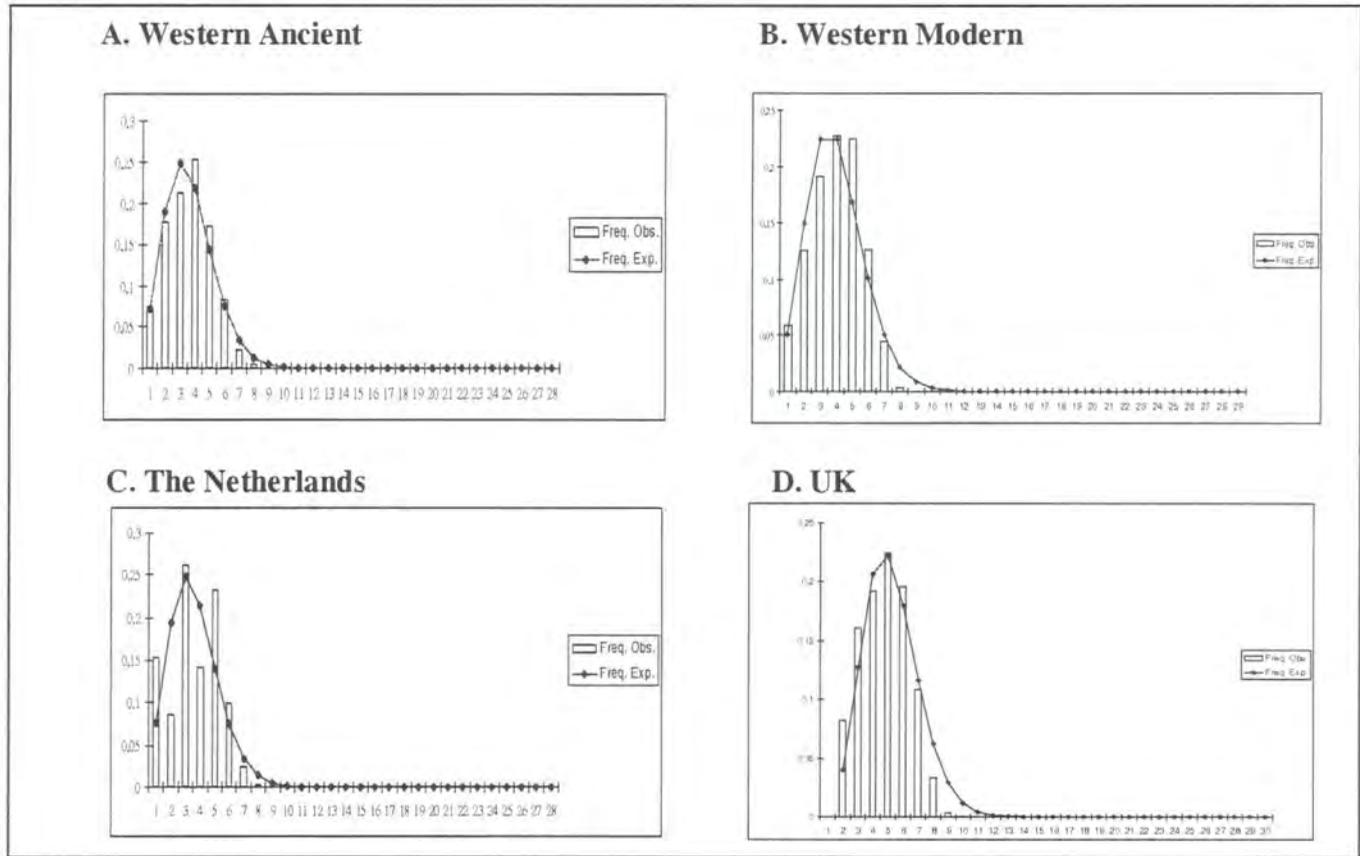
Figure III.6. Mismatch distribution under a model of demographic expansion. The *x* axis shows the number of pairwise differences, the *y* axis shows the frequency of the pairwise comparisons.

Table III.7. Parameters of the mismatch distribution for a model of sudden expansion.

Statistics	Western Ancient N=29	Western Modern N=26	UK N=72	The Netherlands N=35
Tau	2.8301	3.9961	3.0703	3.5176
θ_0	0.0141	0.0000	0.0000	0.0000
θ_1	105.9375	20.4881	20.3906	4.7803
SSD	0.0026	0.0132	0.0016	0.0095
Model (SSD) p-value	0.5600	0.2369	0.6428	0.5972
Raggedness index	0.0322	0.0320	0.0216	0.0287
Raggedness p-value	0.6273	0.4971	0.7603	0.8630
Tajima's D	-1.3455	-0.1667	-1.0567	-0.2350
Tajima's D p-value	0.0759	0.4813	0.1489	0.4537
Fu's Fs test	-13.2814	-4.5163	-4.3200	-3.1684
Fs p-value	0.0001	0.0168	0.0001	0.0552

Table III.8. Calculation of the expansion time ($t = \tau/2\mu$) and effective number of females after demographic expansion ($Ne=\theta_1/2\mu$).

	Demographic Expansion			
	$t = \tau/2\mu = $ years ago			
	Western Modern	**Western Ancient**	**UK**	**The Netherlands**
1*	8022-7220	11327-10194	8703-7832	9971-8973
2	10108	14271	10965	12563
3	23951-16846	33819-23786	25984-18276	29770-20938
	$Ne=\theta_1/2\mu$			
1	270249-300276	52266-58073	52017-57796	12195-13550
2	378348	73172	72824	17073
3	896560-630580	121953-173393	121373-172568	28454-40456

*Numbers correspond to mutation rates (μ) calculated by different authors: 1. Harlin et al. 2003 ($\mu=6.3 - 7.0 \times 10^{-7}$), 2. Ho et al. 2007 ($\mu=5 \times 10^{-7}$), 3. Hayano et al. 2004 ($\mu=2.11-2.99 \times 10^{-7}$).

population toward the western Modern population. The migration rate between western populations was estimated as approximately 0.033-0.079 individuals per generation, using the faster mutation rates (see above) (Figure III.7).

No consistent estimates of current gene flow were obtained using the program Migrate (Beerli 1997-2004; Beerli and Felsenstein 1999), therefore, gene flow (Nm) between populations based on haplotype data was calculated (see Table III.9). Current population sizes were calculated using θ_π and three different mutation rates (see Table III.10); Although some authors have argued that gene flow calculated from Fst are not accurate (see Whitlock and McCauley 1999), it has been also recognized that "in spite of its known limitations, estimates of Fst are often consistent with biologically-informed expectations" (Neigel 2002 p. 168).

2. Genetic variation at microsatellite loci

Of 20 microsatellites screened in *L. acutus*, 11 showed polymorphism with more than 4 alleles. Of the 11 microsatellite loci amplified in tissue samples of *L. acutus* (N=84), one (FCB4) showed evidence for scoring errors due to stuttering; thus this locus was excluded from all subsequent analyses. Teeth samples from the western North Atlantic and the Netherlands were especially problematic to amplify, and the DNA extracted was of low quality, possibly due to the conditions for preserving museum material. Thus, after standardization and evaluation, only five microsatellite loci (IgF1, KwM2a, Texvet5, Texvet7 and Ev94) had good-quality amplification and were successfully amplified in a subset of only 35 samples (14 for The Netherlands and 21 for the western Modern populations). No microsatellite data were available for the western Ancient population.

2.1. Genetic diversity

Ten microsatellite loci were evaluated in Scotland and Ireland populations and five among Scotland, Ireland, The Netherlands and western Modern populations. The genotypic independence between each pair of loci was confirmed using the linkage disequilibrium test. Locus EV94 showed evidence for null alleles in Scotland, Ireland and western Modern populations; locus Textvet5 showed evidence for null alleles in The Netherlands population

Table III.9. Genetic flow (*Nm*) between populations calculated from Fst and φst values.*

	UK	The Netherlands	Western Modern
UK		8.1	inf
The Netherlands	7.0		6.9
Western Modern	27.7	7.4	

*Values below diagonal are from Fst, values above diagonal are from φst; M=1-Fst/2Fst=Nm=individual per generation.

Table III.10. Current population size estimates (*Ne*) from θ_π.

Estimates	UK	The Netherlands	Western Modern
θ_π	2.6838 ± 1.602	2.2420 ± 1.046	2.6379 ± 1.614
$Ne=\theta_\pi/2\mu$			
Ne_1*	6846-7607	5719-6355	6729-7477
Ne_2	9585	8007	9421
Ne_3	15975-22713	13345-18974	15702-22325

*Numbers correspond to evolutionary rates calculated by different authors: 1. Harlin et al. 2003 (λ=6.3 - 7.0 × 10^{-8}), 2. Ho et al. 2007 (μ=5 × 10^{-7}), 3. Hayano et al. 2004 (λ=2.11-2.99× 10^{-8}). Mutation rates per site per year (μ) are equal to λ × generation time (10 years) × number of nucleotides in this study (see Roger and Harpending 1992).
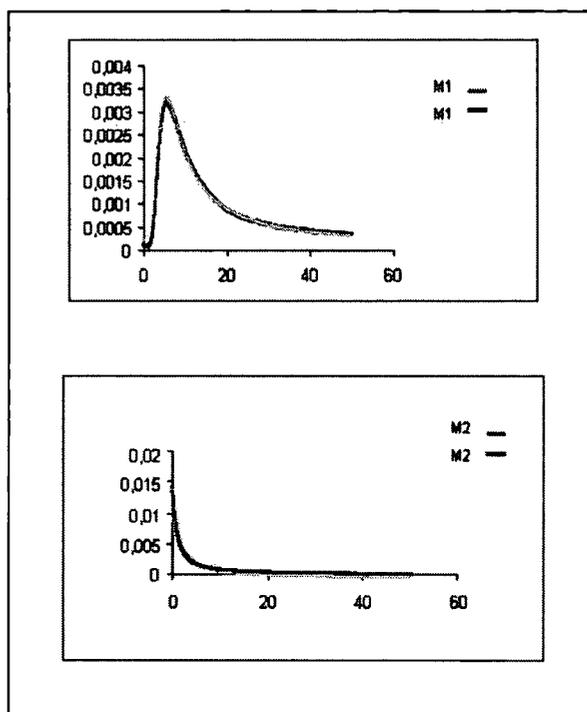
Figure III.7. Posterior probabilities of the migration parameter obtained for western Modern and western Ancient populations with IM program*; x axis shows the migration parameter and y axis shows the probabilities.

*The highest point estimate for M1 was 5.62 (95Lo: 3.175; 95Hi: 46.62). Black lines are values obtained in the first run and grey lines values obtained in the second run. M1 = migration from western Ancient to western Modern and M2 = migration from western Modern to western Ancient.

and locus Texvet7 in the western Modern population. After Bonferroni correction, these three loci (EV94, Textvet5 and Texvet7) also showed a significant deviation from the Hardy Weinberg equilibrium expectations (Table III.11). Excluding loci with null alleles did not affect the estimates of genetic differentiation among populations; therefore all loci were retained for the analysis. Given that only 14 samples were analyzed in The Netherland population, these results should be interpreted with caution.

In the eastern North Atlantic the average gene diversity over 10 loci (N=84) was: 0.7279 ± 0.3871. An average of 10.10 ± 4.228 alleles were found, and the allelic richness per locus based on a minimum sample size of 76 diploid individuals was 9.07. In the western Modern population the average in gene diversity using five microsatellites loci was 0.8091 ± 0.1002 with the mean number of alleles per locus equal to 9.0 ± 3.606. The genetic diversity, allelic richness and number of alleles per locus for each population are shown in Table III.11.

2.2. Population differentiation at microsatellite loci

The Fst values did not show significant differences among regional samples in the eastern North Atlantic using 10 microsatellite loci (Scotland and Ireland)=Fst:0.00422, P< 0.16038. In contrast the Rst statistic showed small, but significant differences between the two regions (Rst: 0.0195 P< 0.03643). The Fst and the Rst statistics also showed contradictory results when all regional samples were compared with only 5 loci (Table III.12).

2.3. Bottleneck analysis

For this test samples from eastern North Atlantic were evaluated first as a single population (Eastern=84) and after by regions (Scotland-Ireland). The populations from the Netherlands and western Modern were not evaluated because of the low number of loci obtained (5) and the restriction in the assumptions used by the program.

The bottleneck analysis showed that the eastern populations (Scotland plus Ireland) are not in mutation-drift equilibrium; nevertheless this deviation was not due to an heterozygosity

Table III.11. Genetic diversity at microsatellite loci in *L. acutus*.

Locus	Population	N	K	R	Ho	He	Fis	Allelic range	G-W statistics
GT136	Scotland	59	9	7.062	0.7759	0.7712	-0.0061	18	0.4737
	Ireland	24	9	8.519	0.9167	0.8351	-0.1000	18	0.4737
IgF1	Scotland	59	12	8.747	0.8305	0.8495	0.0225	26	0.4444
	Ireland	24	9	8.325	0.7500	0.8511	0.1210	18	0.4737
	The Netherlands	13	8	5.487	0.7857	0.8518	0.0800	16	
	western Modern	20	6	5.511	0.6500	0.8115	0.2030	13	
KWM2a	Scotland	59	7	5.497	0.7288	0.6716	-0.0860	26	0.2593
	Ireland	24	10	8.380	0.7083	0.7846	0.0991	34	0.2857
	The Netherlands	10	7	4.263	0.6364	0.7662	0.1760	22	
	western Modern	12	8	5.602	0.7692	0.8677	0.1180	24	
DO8	Scotland	58	6	4.538	0.5345	0.6036	0.1154	10	0.5454
	Ireland	25	5	4.262	0.2000	0.5657*	0.6512**	10	0.4545
D22	Scotland	58	6	5.543	0.8965	0.7610	-0.1799	10	0.5454
	Ireland	20	6	5.977	0.7500	0.7846	0.0452	10	0.5454
GT011	Scotland	58	6	4.182	0.3220	0.3422	0.0593	12	0.4615
	Ireland	17	4	4.000	0.2353	0.2228	-0.0579	6	0.5714
Texvet 5	Scotland	58	5	3.468	0.5690	0.5633	-0.0102	8	0.5556
	Ireland	22	4	3.725	0.5000	0.5444	0.0833	6	0.5714
	The Netherlands	12	4	3.340	0.3846	0.7015*	0.4620**	16	
	western Modern	21	5	2.988	0.6190	0.6353	0.0260	24	

Table III.11. Continuation. Genetic diversity at microsatellite loci.

Locus	Population	N	K	R	Ho	He	Fis	Allelic range	G-W statistics
Textvet 7	Scotland	59	17	12.056	0.9153	0.8750	-0.0464	48	0.3470
	Ireland	25	15	12.499	0.8800	0.8816	0.0019	42	0.3488
	The Netherlands	10	3	3.000	0.6000	0.7316	0.1880	44	
	western Modern	19	13	6.385	0.6842	0.8563*	0.2050**	46	
Ev94	Scotland	59	13	10.293	0.7797	0.8560	0.0899	26	0.4815
	Ireland	23	12	10.501	0.5217	0.8261	0.3737**	30	0.3871
	The Netherlands	13	12	6.853	0.9230	0.8984*	-0.029	44	
	western Modern	19	12	6.238	0.6315	0.8748*	0.2840**	49	
EV37	Scotland	59	12	8.363	0.7931	0.8327	0.0479	26	0.4444
	Ireland	23	8	7.472	0.6956	0.8222	0.1569	14	0.5333
Overall eastern (10 microsatellites)	Scotland		9.3	6.975	0.7145	0.7126	-0.003	21	0.4558
	sd		4.001		0.1853	0.1696		11.533	0.0882
	Ireland		8.2	7.366	0.61577	0.7118	0.137**	18.8	0.4645
	sd		3.584		0.24748	0.2076		11.839	0.0926
Overall western–eastern (5 microsatellites)	The Netherlands		9	2.294	0.6659	0.7899	0.163**	31.200	0.3183
	sd		3.162		0.2030	0.0827		13.659	0.1118
	Western Modern		9.000	2.672	0.6708	0.8091	0.175**	31.2	0.3136
	sd		3.606		0.0602	0.1002		13.934	0.1392

Fis: Degree of random mating as in Weir and Cockerham (1984). Significant values of Fis (P<0.05) are labelled with two asterisks(**); N: number of samples; K: number of alleles; R: Allelic Richness; G-W: Garza-Williamson statistics; Ho: Observed heterozygosity; He: Expected Heterozygosity. *Loci that showed significant departure from HW equilibrium expectations.

Table III.12. Fst and Rst values in populations of *L. acutus.* *

	Scotland	Ireland	The Netherlands	Western Modern
Scotland		0.00422*	0.0220	**0.0328**
Ireland	**0.0195***		0.0294	0.0192
The Netherlands	**0.0164**	0.0085		**0.0375**
Western Modern	0.0043	0.0131	**0.0339**	

Values below the diagonal are Rst values; above the diagonal, Fst values. Values with asterisk (*) were computed using 10 microsatellites loci. Values in bold are significant at 0.05 level.

excess as expected in populations that have been affected by recent bottleneck, but rather to an heterozygosity deficiency. When separate analyses by region were performed, 1 locus with heterozygosity deficiency and 9 loci with heterozygosity excess were found under the IAM model (P< 0.04385) suggesting recent bottleneck in Scotland; however given that the model for recent bottleneck should be significant under the three mutation models this result should be interpreted with caution. No evidence was found in any of the three models to suggest a bottleneck for the group from Ireland. In contrast to the finding with the bottleneck program the low M-Garza values suggested bottleneck in both populations (Table III.11).

DISCUSSION

1. Genetic variability in Lagenorhynchus acutus

A total of 162 mitochondrial control DNA sequences were obtained from *L. acutus* throughout its geographic range. They were evaluated in combination with microsatellite loci in order to address questions about the processes that have influenced the evolution, differentiation and genetic diversity of this species in the North Atlantic.

The mtDNA genetic diversity found in this species (0.847 to 0.955) contrasts with its low nucleotide diversity (0.0086 to 0.01); this nucleotide diversity range is lower than has been found in other dolphin populations, which sometimes show values higher than 0.1 (see Pichler and Baker 2000). The pattern of high genetic diversity and low nucleotide diversity as found in *L. acutus* is normally attributed to population expansion, which creates an excess of haplotypes differing by one or a few mutations (Rogers and Harpending 1992). To establish whether or not the pattern of genetic diversity found in this species is due to population expansion, the mismatch distribution was analyzed and is discussed below.

1.1. A possible effect of ice ages on the genetic variability of L. acutus populations: evidence from the mismatch distribution and neutrality tests

The results from this study show a clear pattern of unimodal shape in the mismatch distribution in all regional populations of this species that were studied. This can be explained either by a rapid increase in population size followed by a period of large and constant population size, or due to a selective sweep. This is where natural selection acting in favour of one mitochondrial genotype results in a rapid increase in the number of individuals carrying the favoured mitochondrial type (see Roger and Harpending 1992; Slatkin and Hudson 1991). Although it has been recognized that mtDNA can be affected by natural selection, the consistency of the expansion signal among populations of *L. acutus*

supports the idea of a general effect explaining the patterns found in this species rather than selection (less likely to affect all populations the same way).

It is well known that the influence of the ice ages on climate was long term and continuous in temperate regions (Hewitt 2000). Therefore most species in the highest latitudes were affected by the postglacial expansion after the retreat of the last glacial (Qu et al. 2005). Studies in several species in the Northern Hemisphere have revealed that this postglacial expansion into new territories was important in the geographic distribution of species genomes and left an important signature in their DNA. A rapid expansion from refugial populations involved several bottlenecks with progressive loss of allelic diversity among populations in the postglacial colonized regions (see review in Hewitt 2000).

The effects of past climate changes have been quite well studied in terrestrial animals (e.g., Lee-Yaw et al. 2008; Peters et al. 2005; Galbreath and Cook 2004; Michaux et al. 2003) and recently in a few marine species. Some studies in marine species have shown that their population structures and their genetic variability were also affected by the isolation in refugia and posterior recolonizations during glaciated epochs (e.g., Wares 2002; Addison and Hart 2005; Bigg et al. 2008; Chevolot et al. 2007; Vermeij 2005). For example Pastene et al. (2007) suggest that the differentiation between northern and southern populations of common minke whale (*Balaenoptera acuturostrata*) was mainly occasioned by the glacial cycles and intermittent warming periods during the Pliocene-Pleistocene epochs. The phocine seals (Genus *Phoca*) have been also reported among the species whose diversification, colonization patterns and genetic structure have been led by the periodic changes in the sea level, ice cover and temperatures in the Northern Hemisphere (Palo 2003; Goodman 1998).

The results of this study also suggest that the demographic history and genetic variability of *L. acutus* were affected by the strong Pleistocene climatic changes; however this premise is highly dependent on the accuracy of the mutation rates that are used to date the demographic events. Assuming that the fastest mutation rates estimates by Harlin et al. 2003 and Ho et al. 2007 are accurate (6.3-7.0×10^{-7} and 5.0×10^{-7} respectively), the time for a possible expansion in populations of *L. acutus* could be placed after the last glacial

maxima in the late Pleistocene, early Holocene period, with values ranging from 14000 years ago to 7000 years ago. Interestingly, the oldest estimates of expansion time and largest female effective population sizes were found in the western North Atlantic (~14000 years ago to 10000 years ago in the western Ancient population and ~378000 to ~ 300000 females in the modern Western population). These values possibly suggest an expansion origin in this region. However this observation is not strongly supported as only two populations from the western North Atlantic were evaluated. Moreover, because the parameter for the time of splitting did not reach convergence in any of the populations that were analyzed –using either Mdiv or IM program, there may have been ongoing dispersal through to the present among these populations, or greater power may be required to resolve a division time.

2. Population structure in L. acutus

To address the question about genetic differentiation among populations of L. acutus across its geographic range, the Fixation Index was evaluated with both mitochondrial and nuclear loci.

No differentiation between the eastern and the western North Atlantic was found for the mtDNA marker (ϕst=-0.0005 P<0.4155; Fst=0.0126 P<0.0967). These results partially agree with the results reported by Mikkelsen and Lund (1994). Those authors did not find any craniometrical differences among samples from L. acutus on either side of the North Atlantic. In contrast, the data in this study suggest the existence of one isolated population in the southern North Sea (The Netherlands), which is different from the other populations and also shows a distinct signal for historical demographics. The results differ to some extent between the nuclear and mitochondrial molecular markers, although sampling effects may be important in the relatively small sample sizes applied to the microsatellite DNA analyses.

- *The eastern North Atlantic*

In this study evidence was found for subdividing the *L. acutus* population into at least two population stocks in the eastern North Atlantic. Individuals sampled in the southernmost part of the species's geographic range (The Netherlands and southern North Sea) showed significant genetic differentiation (for both mitochondrial and nuclear DNA) from individuals in the Northwest (mainly Scotland).

This differentiation may be a function of the habitat preferences of *L. acutus*, which is mainly a pelagic species with preferences for deep waters ranging from 100-500 m, and is rarely found in the southern North Sea. Individuals found in this region could belong to an isolated population that has reduced its genetic flow with oceanic populations due to the geographic and geological characteristics of the southern North Sea. This habitat is considered an unfavorable environment for oceanic species due to its intricate system of sand banks, mudflats, sandy islands and estuaries (Das et al. 2003).

On the other hand the isolation of the Netherlands population may perhaps be explained by the changes in sea level that took place during the glaciated epochs and the extension of the ice sheet cover in Northern regions. It is well known that during the Pleistocene era the ice sheet covered most part of the North Sea, with the exception of the Southern North Sea (see Siegert and Dowdeswell 2004; Fig 1). Therefore, it is possible that individuals of *L. acutus* previously distributed in this area became isolated from the rest of populations during the Pleistocene epoch. The parameters in the mismatch distribution calculated for The Netherlands population agrees with this premise, the analysis suggests a demographic expansion in this population starting around ~12500 and ~9000 years ago (see Table III.7) after the last glacial maxima and also suggest that the effective size of this population was approximately fourfold lower than the other *L. acutus* populations.

A study increasing the number of samples from this region would help to address questions about the origin of this population and the processes that have influenced its isolation and survival in an area considered not suitable for pelagic species.

- *The Western North Atlantic*

Although no differentiation was found between the western Modern and eastern populations (UK), significant differentiation was found between the two western populations (φst: 0.0862 P<0.0051 Fst=0.0438 P<0.0001). This result is somewhat unexpected; as Mikkelsen and Lund (1994) using craniometrical analysis in individuals from a similar distribution range did not find evidence to suggest the existence of differentiated populations in the western North Atlantic (see Mikkelsen and Lund 1994; Figure 5); and this contradicts expectations based on isolation by distance. In addition, several population stocks have been proposed in this region: Gulf of Maine, Gulf of St. Lawrence and Labrador Sea, based mainly on sightings; strandings and incidental takes (Palka et al. 1997); however, the existence of these stocks has not been evaluated yet with molecular approaches.

Assuming that the degree of differentiation found between western Ancient and western Modern populations, is a reflection of the true differences between these populations, several possible hypotheses could explain this. First, Nova Scotia has been proposed to be a northern Pleistocene refugia in the western North Atlantic (Wares 2002), based on the position of glacial margins and biogeographical pattern of faunal diversity centered in this region. It is also possible that the relatively shallow bay of Fundy, connected to the Gulf of Maine, would have been protected from the exposed Atlantic coast line and provided a suitable habitat for Northern populations during glaciated epochs (in Adams et al. 2006). Following this premise it is likely that the differentiation found in this study between western Ancient and western Modern populations could be a reflection of past demographic history of this region. Further sampling around the western cost of the North Atlantic, from North to South will help to clarify this issue.

On the other hand if there are no differences among groups in the western North Atlantic, as proposed by Mikkelsen and Lund (1994), the differentiation between the two populations could be an indication of changes in the genetic composition of the population over the last 100 years. However, given that it is not clear whether or not unique haplotypes found in the

western Ancient population are extinct or are still present, but were not sampled in the modern western North Atlantic, a larger sample set would help resolve this question.

3. Testing the hypothesis of a recent bottleneck in L. acutus

To evaluate whether the loss of genetic diversity in *L. acutus* is consistent with the hypothesis of population decline due to a recent bottleneck, the M-Garza statistics and the sign test in the bottleneck program, were used.

Little was provided by this study to claim a recent reduction in the population sizes of *L. acutus* populations due to anthropogenic activities. Under the assumptions of the bottleneck program only the Scotland population showed heterozygosity excess with the IAM mutational model, which is only a weak signal (see Cornuet and Luikart 1996). However, the M Garza statistic showed evidence for a bottleneck in all populations. This statistic is capable of detecting bottlenecks that are older, but still typically younger than the time-frame indicated by the mismatch distributions (Garza and Williamson 2001).

CHAPTER IV: PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE WHITE-BEAKED DOLPHIN (*LAGENORHYNCHUS ALBIROSTRIS*)

INTRODUCTION

1. Distribution and use of habitat

The Atlantic white-beaked dolphin (*Lagenorhynchus albirostris* Gray, 1846) is the most northerly member of the genus *Lagenorhynchus* and is restricted to temperate and sub-polar seas along the North Atlantic (Leatherwood et al. 1976). Its distribution extends from southern New England, North to south-western Greenland and the Davis Straits in the western North Atlantic to northern Scotland, Norway, Iceland, the Greenland Sea and Central-West Greenland in the eastern North Atlantic (Reeves et al. 1999; Leatherwood et al. 1976). It is also frequently sighted in British and Irish waters, as well as in the North-Central North Sea (Evans 1992) (Figure IV.1).

L. albirostris is considered a typical coastal species (Evans 1992; Mikkelsen and Lund 1994); and its spatial distribution and relative abundance along the Continental Shelf and coastal areas has been highly correlated with physical factors such as the presence of high marine productivity, sea temperatures (<12°C) and water depths (<120 m); as well as with ecological factors such as prey abundance, competition with other species and seasonal migration (MacLeod et al. 2007, Weir et al. 2007; Simard et al. 2006). For example, high relative abundance of this species has been reported from June to August in the north-western North Sea (Aberdeenshire coasts) with a clear peak during August, correlated with a high abundance of prey (e.g., mackerel) (Weir et al. 2007). Baumgartner et al. (2006) also found seasonal changes in the relative abundance of this species throughout the year in North-east Scotland and farther North; however, in contrast with the study by Weir et al. (2007) these authors did not find the species in the studied area from May to October, but suggested its presence in the region during the winter season. In previous studies Northridge el al. (1995) also reported variation in the distribution of this species, possibly related to feeding and breeding habitats.
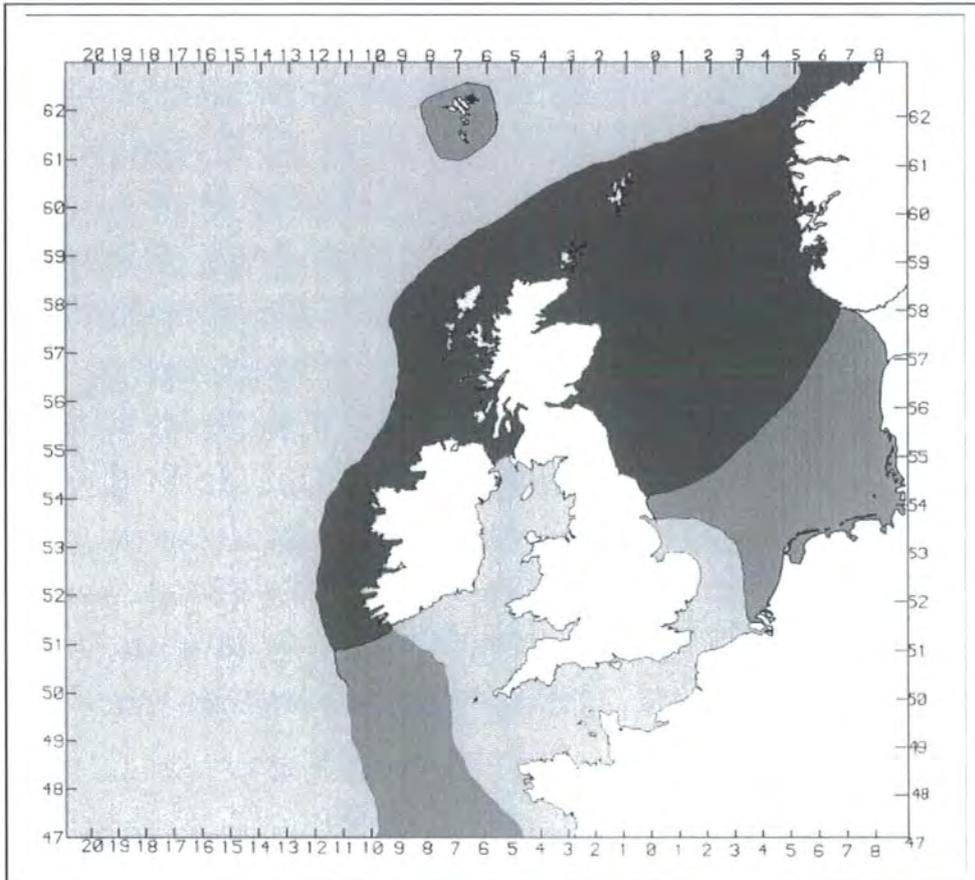
Figure IV.1. Distribution of the White-beaked dolphin (*Lagenorhynchus albirostris*) in the UK and adjacent northern European waters.

Source: Sea Watch Foundation data and knowledge of the ecology of the species. Courtesy: Prof. Peter Evans from Sea Watch Foundation. Status: Regular (dark shading); Occasional (intermediate shading); Casual/Absent (light shading).

Although seasonal migrations have been reported as an important factor in the relative abundance of *L. albirostris*, it is not clear whether this migration occurs between close coastal areas or between offshore-coastal regions (Weir et al. 2007; Northridge et al. 1995). Weir et al. (2007) suggested that migration could be related to reproductive behaviour because the majority of groups sighted on the coast had a high composition of calves and juveniles. Furthermore, Evans (1991) considers that the species gives birth offshore in the northern North Sea; thus it is likely that offshore-coastal migration can occur in *L. albirostris* after calving.

Use of habitat, seasonal movements and differential migration between males and females have been reported as important factors influencing the extent of genetic diversity in cetacean populations (e.g., Cassens et al. 2005, Hayano et al. 2004; Hoelzel 1998). Frequently, individuals that converge in specific areas belong to different social groups that are not easily detectable using demographic data; thus the study of genetic diversity in these temporal associations together with accurate ecological data can provide a better understanding about the ecology of populations and generate information about primary areas for conservation of the species (Fredsted et al. 2005; Rosa et al. 2005; Hoelzel 1998; Neigel 1997). Moreover, molecular data can give new insights into the evolutionary processes influencing the genetic diversity of coastal populations and to recognize the extent of genetic variation available to allow their adaptation to local environments (Lee-Yaw et al. 2008).

In this chapter two molecular markers (mtDNA and microsatellites) are used in order to test the relationships between genetic diversity, migrational processes and use of habitat in populations of *L. albirostris* in the North Atlantic. Analyses of the differences between the genetic composition at the bi-parental (microsatellites) and matrilineal (mtDNA) levels will allow a better understanding of the effects of social behaviour on the genetic diversity of this species and of the principal evolutionary and ecological processes maintaining this diversity.

2. Population structure and phylogeographic patterns

Similar to other members of the genus *Lagenorhynchus* (e.g., *L. acutus*) little is known about the population structure of *L. albirostris* in the North Atlantic and North Sea. Suggestions about population stocks in this species have been mainly drawn from short-term, localized sightings surveys (e.g., Evans and Hammond 2004; Hammond et al. 2002; Northridge et al. 1995). For example, Northridge et al. (1997, 1995) suggested a possible stock separation in the eastern North Atlantic, with individuals in the North Sea and around the British Isles being a distinct population from those found in the North-west. In contrast, Mikkelsen and Lund (1994), using skull features, did not find any significant differentiation among individuals from the eastern North Atlantic; but strong evidence was found to suggest the existence of at least two separate stocks in this species: one in the eastern and one in the western North Atlantic.

Studies in marine and terrestrial mammals have shown that the evolution of population structure can be influenced by a number of different factors such as dispersion capacity, ecological specialization, physical barriers, reproductive behaviour and social structure (Dalebout et al. 2006; Fredsted et al. 2005; Hoelzel et al. 1998a). For example, the complex pattern of genetic differentiation (mainly at the intra-population level) found in several coastal populations of delphinids, has been highly associated with differences between spatial distribution of males and females, high fidelity for feeding or breeding areas, habitat specialization and mixed assemblages – the temporal association of individuals from different populations and lineages in an specific area – (Gaspari et al. 2007; Amaral et al. 2007; Querouil et al. 2007; Natoli et al. 2005; Cassens et al. 2005; Krutzen et al. 2004; Hayano et al. 2004; Gladden et al. 1999; Hoelzel 1998; Hoelzel et al. 1998a).

In addition, historical demographic processes, in particular the Pleistocene glaciations in the Northern Hemisphere, have also been claimed as an important factor leading to genetic differentiation among marine species and populations, promoting speciation and influencing the distribution of lineages in coastal areas (e.g., Haney et al 2007; Adams et

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al. 2006; Rosa et al. 2005; Harlin et al. 2003; Hayano et al. 2004; Hewitt 2000; Reeb and Avise 1990). It has been hypothesized that during these climatic changes the distribution range of many marine species was restricted due to the creation of physical barriers (e.g., changes in sea level and sea temperatures) that reduced gene flow, even among proximate populations, and increased the effect of evolutionary forces such as genetic drift and selection (Haney et al. 2007; Hewitt 2000, 1996; Avise et al. 1998). After the end of the ice age, populations that were previously limited in small and specific areas probably expanded following a stepping stone model (dispersal occurring between neighbouring demes), or experienced a sudden demographic expansion, which could explain the different patterns of genetic diversity found in contemporary northern populations (Wegmann et al. 2006; Excoffier 2004; Ray et al. 2003; Austerlitz et al. 1997; Rogers and Harpending 1992; Slatkin and Hudson 1991).

In this chapter I will evaluate two main hypotheses regarding the influence of past events in the evolutionary history and genetic structure in *L. albirostris*, as well as the processes leading to genetic differentiation. Firstly, if this species has been affected by the climatic changes in the North Atlantic during the last glaciations, low genetic diversity (due to reduced population size in refugia) should be expected, as well as a clear genetic signature for either an expansion in range or a sudden demographic expansion. Secondly, given that the species is primarily restricted to coastal areas, it is expected that the deep seas between the western and eastern North Atlantic and between some places in the eastern North Atlantic form an effective barrier for its dispersion, and thus some degree of genetic differentiation should be expected across deep sea barriers.

The foregoing hypotheses will be addressed analyzing the mtDNA d-loop region and microsatellite markers, using Bayesian statistics and coalescence approaches to provide inference of population dynamics and gene flow (Hey and Nielsen 2004; Excoffier 2004; Nielsen and Wakeley 2001).

3. Population sizes and principal trends in L. albirostris

Information on population sizes of *L. albirostris* is scarce, and no exhaustive studies have been carried out either in the eastern or western North Atlantic (see Weir et al. 2007; Waring et al. 2007; Waring et al. 2006; Kinze et al. 1997). Øien (1996) consider that the species is relatively abundant (several thousands) in Icelandic waters and in the north-eastern Atlantic including the Barents Sea, the eastern part of the Norwegian Sea and the North Sea north of 56°N (IUCN 2007). Hammond et al. (2002) suggested that the average population of *L. albirostris* in the North Sea and Channel could be around 7856 individuals.

The species is considered less abundant in north eastern areas compared with the western North Atlantic (Lien et al. 2001). However, the only population data available for the western North Atlantic reported a population estimate of 5500 dolphins in Canadian waters (Lien et al. 2001; Alling and Whitehead 1987) and one estimate of 573 animals in waters off Cape Cod in 1982; further surveys in the latter area and over the US Atlantic continental shelf did not report any sighting for this species (Waring et al. 2006).

It is well known that bycatch in fisheries, over-fishing of prey, degradation or loss of coastal habitats and pollution, among others, are the principal causes leading to vulnerability or extinction in coastal species (Cassens et al. 2005, Rosel et al. 1999). These events are often translated into a reduction in effective population size, and the resulting loss in diversity that can affect the capacity to respond to environmental changes (Cassens et al. 2005; Pichler and Baker 2000; Rosel et al. 1999; Castello 1996).

Even without detailed census data, it is possible to draw inference from trends identified in other coastal delphinids populations. For example, small scale hunting has been and is still common in some countries such as Norway, the Faroe Islands, Greenland, Iceland, and Canada (Lien et al. 2001; Reeves et al. 1999; Jefferson et al. 1993). Alling and Whitehead (1987) have claimed that approximately 366 individuals of *L. albirostris* are killed for subsistence in northern and southern Labrador each year. Moreover, individuals

of this species are frequently taken in a variety of fishing gear throughout their range (IUCN 2007).

In order to understand the evolutionary history of *L. albirostris* populations, it is important to recognize the processes that have brought about fluctuations in its current or historical population sizes. This will make it possible to infer how these populations are coping with these processes and then elucidate the principal trends for their long-term survival.

In addition to the hypotheses presented in previous sections, the issue of whether or not the population sizes in *L. albirostris* in the eastern North Atlantic have declined recently will also be tested in this chapter, using microsatellite data. Possible external factors causing a deviation from the mutation-drift equilibrium expected in constant populations will be evaluated and ancestral and current population sizes will be estimated in order to provide data for the effective conservation of this species.

MATERIALS AND METHODS

1. Sampling collection

A total of 167 samples of *L. albirostris* were collected from different localities in the eastern and western North Atlantic through cooperative agreements with several institutions (see previous chapter). Samples from the western North Atlantic are from Canada, while samples in the eastern North Atlantic belong to different regions: North Sea, English coast, The Netherlands and Norway. Samples from Norway were obtained under agreement with Dr. Arne Bjorne from the Institute for Marine Research in Bergen. The numbers of samples and their localities are shown in Table IV.1 and Figure IV.2.

2. Mitochondrial d-loop region: DNA extraction, amplification and sequencing

Total genomic DNA was extracted from tissue following the procedure recommended by Hoelzel and Green (1998). Tooth sample extractions, PCR amplification and DNA sequencing were performed using the protocols and conditions described in chapter III.

3. mtDNA analyses

Samples were divided into three regions in the eastern North Atlantic: The Netherlands (N=38), Norway (N=33) United Kingdom –UK (N=38) and one region in the western North Atlantic –WNA (N=13) that includes seven from Canada and 6 samples from the NCBI GenBank database (accession No: EF092928, EF092930, EF092929, AJ554061, EF092931 and EF092932).

The extent of genetic variation in the control region was assessed by examining the haplotype (h) and the nucleotide diversity (π). The degree of genetic differentiation among geographic samples was analyzed using an Analysis of Molecular Variance (AMOVA).

Table IV.1. Samples obtained from *L. albirostris* in each region.

COUNTRY	COUNTY	N	LOCATION	n
SCOTLAND	GRAMPIAN	10	Aberdeen Harbour	3
			Balmedie	2
			Clubbie Craig, Fraserburgh	1
			Crovie, Banff	1
			Forvie Nature Reserve	1
			Perthudden, Collieston	1
			Fraserburgh Beach	1
	FIFE	1	Grangemouth	1
	HIGHLAND	12	Glenelg	1
			Brora	1
			Dunnet Bay	1
			Reiff	1
			Ard Neackieh Erribol	1
			Geanies,Hilton Of Cadboll	1
			Clash, Kinlochbervie	1
			Thurso Beach	1
			Raasay Forest, Raasay	2
			Scrabster Beach	1
			Dunnet Beach, Near Thurso	1
	LOTHIAN	3	Drum Sands, Edinburgh	1
			Gosford Sands	1
			Longniddry	1
	ORKNEY	2	Scara Brae, Mainland	1
			Brough Of Birsay	1
	SHETLAND	1	Basta Voe, Yell	1
	STRATHCLYDE	1	Glenmhor Bay	1
	WESTERN ISLES	1	Ardivacher, South Uist	1
Subtotal Scotland		31		31

Table IV.I. Continuation. Samples obtained from *L. albirostris* in each region.

NORWAY		41		41
ENGLAND				
	Devon	1	Brixham Harbour	1
	Durham	1	Hartlepool	1
	Humberside	5	Withernsea	1
			Low Skirlington	1
			Hornsea	1
			Bridlington	1
			Spurn Point	1
	Lincolnshire	1	Sandilands	1
	Norfolk	1	Gorleston-on-Sea	1
	North Yorkshire	1	Cayton Bay, Osgodby	1
	Northumberland	1	Blyth	1
	Suffolk	1	Sizewell	1
	Tyne and Wear	2	Sth. Shields	1
			Whitley Bay	1
Subtotal England		14		14
THE NETHERLANDS		82		82
DENMARK		3		3
ICELAND		2		2
NORTH SEA		4		4
IRISH SEA		8		8
CANADA		13		13
Total		167		167

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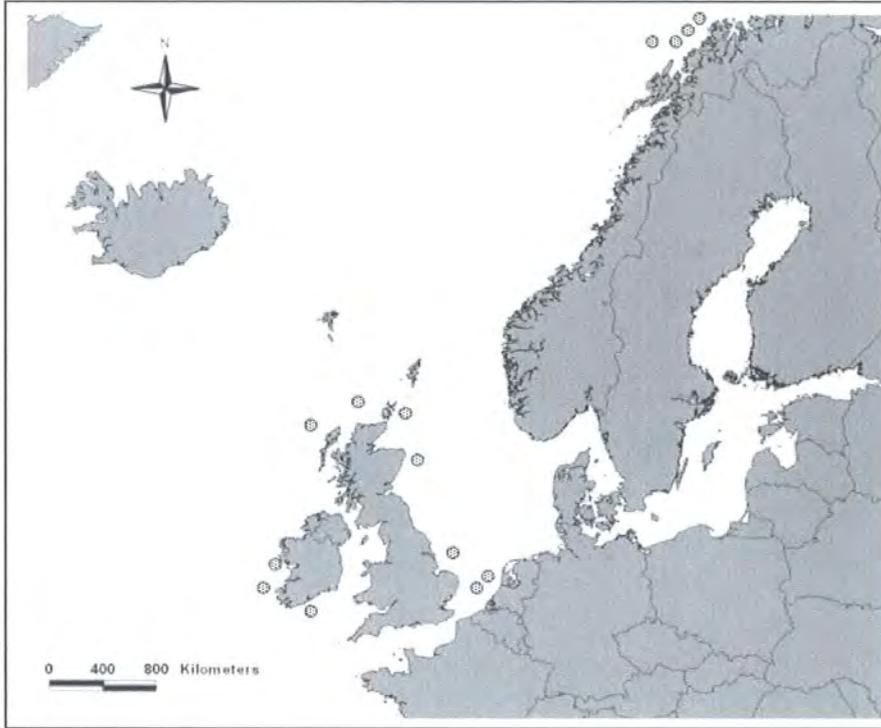
Figure IV.2. Sampling localities in the eastern North Atlantic.

Note: Samples from the western North Atlantic belong to Canada, but specific locality is unknown.

Gene flow between populations and current population sizes (Ne) were assessed as described in chapter III. All analyses were performed using Arlequin v 3.11 (Excoffier et al. 2007) and DNAsp v 4.0 (Rozas et al. 2003).

A neighbour-joining tree and a median-joining network tree for the complete set of mtDNA haplotypes were generated in order to evaluate the phylogenetic relationships among haplotypes. Analyses were conducted using PAUP* v. 4.0b10 (Swofford 2002), MEGA v. 4.0 (Tamura et al. 2007) and Network (Bandelt et al. 1999).

The demographic history of the populations was assessed using the mismatch distribution (Rogers & Harpending 1992). The fit to the model was evaluated using the sum of square deviations (SSD) between the observed and the expected mismatch and the raggedness index (r) of the observed distribution (Harpending 1994; Schneider and Excoffier 1999). Sudden expansion was also tested using the Fu's test (Fu 1997) and Tajima's tests (Tajima 1989b) in the program Arlequin v 3.11 (Excoffier et al. 2007). The time of the expansion, the effective population size after the expansion ($\theta=2Ne\mu$), as well as other population parameters were estimated using a generation time of 10 years (Ferrero and Walker 1996) and different mutation rates estimates by other authors (Ho et al. 2007; Hayano et al. 2004; Harlin et al. 2003) (Refer to chapter III for details).

Given that populations of *L. albirostris* did not show a clear signal for demographic expansion (see results), a model of spatial expansion was evaluated. The programme Arlequin 3.11 (Excoffier et al. 2007) was used to derive the expected mismatch distribution under the continent-island model (equivalent to an infinite island model) (see Excoffier et al. 2007), which assume that genes were sampled from a single deme, belonging to a population subdivided into an infinite number of demes of size N that exchange m migrants with other demes. Three parameters of spatial expansion were estimated: τ, $\theta=\theta_0=\theta_1$ assuming $N=No$ *and* $M=Nm$ using a least-square method. The fit to the model was tested by coalescent simulations, assuming an instantaneous expansion under the continent-island model as describe by Excoffier (2004).

The IM (Hey and Nielsen 2004) and Mdiv (Nielsen and Wakeley 2001) programs were used to infer several population parameters under a model of isolation-with-migration. See chapter III for further description of the analyses. Three pairs of populations were compared: UK-Norway, WNA-Norway, UK-WNA. For these analyses, samples from the Netherlands and UK were combined in a single population, given that the F_{ST} values showed no significant differences between these two populations (see results). The Mdiv programme was run twice using 5×10^6 and 10×10^6 chains and a burn-in of 10% (500000 and 1000000 respectively) as recommended by the author. The IM program was run to compare the closest populations (UK-Norway) with the same parameters that were used for *L. acutus* (see chapter III).

4. Microsatellite loci

- ### Testing heterologous microsatellites

A total of 26 microsatellite loci from the literature were titrated for use with *L. albirostris* and a subset chosen for further screening (see Table IV.2). For specificities about the amplification and PCR conditions for loci that were also screened in *L. acutus* (Ev94, Texvet7, Gt136, D22, FCB4, Ev37 and KwM2a), refer to Chapter III. Loci Di19, Di 24, Di 9 and Di 47 were amplified using the PCR conditions described by Cassens et al. (2005).

- ### Microsatellite library for L. albirostris

As many of the screened heterologous loci showed no or low polymorphism in *L. albirostris*, a species-specific microsatellite library was built using the protocol described by Carleton et al. (2002).

Genomic DNA was isolated from tissue of one individual of *Lagenorhynchus albirostris* (~10 ug), and was restricted with the SAU3A enzyme. DNA fragments 400-750 bp in size were cut out after electrophoresis in 1% low melting-point agarose. Linkers A (5' GCGGTACCCGGGAAGCTTGG), and B (5' ATCCCAAGCTTCCCGGGTACCGC)

Table IV.2. Microsatellite-loci standardized in *L. albirostris.*

	Locus	Annealing Temperature °C	Alleles sizes	Reference
1	415/416	47	Monomorphic	Amos et al. 1993
2	D08	54	107-109	Shinohara et al. 1997
3	D22*	49	115-123	Shinohara et al. 1997
4	D28	52	Monomorphic	Shinohara et al. 1997
5	EV37*	48/57	195-197	Valsechi and Amos 1996
6	FCB4*	55	171-177	Buchanan et al. 1996
7	FCB5	55	138-142	Buchanan et al. 1996
8	GT011	59	125-131	Bérubé et al. 1998
9	GT101	43	Monomorphic	Andersen et al. 2001
10	GT136*	52	101-105	Andersen et al. 2001
11	Igf1	59	Monomorphic	Andersen et al. 1997
12	KWM12a	54	Monomorphic	Hoelzel et al. 1998b
13	KWM2a*	49	133-141	Hoelzel et al. 1998b
14	KWM1b	50	183-189	Hoelzel et al. 1998b
15	TAA031	55	Monomorphic	Palsbøll et al. 1997
16	Textvet5	52	193-201	Rooney et al. 1999
17	Textvet7*	59	147-159	Rooney et al. 1999
18	EV5	48/56	Monomorphic	Valsechi and Amos 1996
19	EV94*	48/56	240-256	Valsechi and Amos 1996
20	Lobs Di9*	61	105-112	Cassens et al. 2005
21	LobsDi19*	61	101-114	Cassens et al. 2005
22	LobsDi21	61	Monomorphic	Cassens et al. 2005
23	LobsDi24*	58	120-128	Cassens et al. 2005
24	LobsDi39	58	Monomorphic	Cassens et al. 2005
25	LobsDi47*	53	107-116	Cassens et al. 2005
26	LobsTT6	61	Monomorphic	Cassens et al. 2005

Microsatellites that were selected after standardization and evaluation are labeled with asterisk (*).

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were ligated onto DNA fragments using T4 DNA ligase; one pre-hybridization PCR amplification was performed using linker A. For enrichment, the DNA was denatured and hybridized to a biotinylated microsatellite probe (B-ATAGAATAT $(CA)_{16}$) and then captured with streptavidin magnetic beads. After purification the DNA was PCR amplified using the PCR condition from Carleton et al. (2002). The hybridized DNA was cloned into a pCRTOPO 2.1 vector (Invitrogen, Inc), and the ligation reaction was utilized to transform TOP10 electro-competent cells and plated.

A blue-white screening was used to select positive colonies, and the insert size was tested by PCR using two universal primers (M13 reverse and forward) and one microsatellite-specific primer (5'TGT GGC GGC CGC $(TG)_8$ – 3'). The positive colonies were grown in overnight cultures of 6-8 ml at 37°C; the culture was miniprepped using a GeneElute^{TM} Plasmid Mini-prep Kit (Sigma). Each clone was cut with ECORI and sequenced in one direction in an ABI 377. After sequencing, clones that showed associated microsatellites were used to design microsatellite-specific primers using the Oligo programme.

The new primers were PCR amplified using 20-50 ng of DNA in a final volume of 20 ul. The reaction mix contained 200nM of each primer, 0.5-1.5mM MgCl2, 0.1mM-0.36mM of dNTPs and 0.2U of Taq polymerase. PCR conditions were as follows: Primer denaturation at 95°C for 5 min, 35 cycles at 94°C for 45 sec, 1 min 30 sec at locus-specific annealing temperature, extension at 72°C for 1 min 30 sec followed by 5 min final extension. Primer sequences, annealing temperatures and magnesium concentrations are shown in Table IV.3. For further details of microsatellite analyses, see Materials and Methods section from Chapter III.

RESULTS

1. Genetic variation at the mtDNA control region

After sequencing, 122 samples (including samples from the NCBI GenBank data base) were analyzed using two fragments of the control region in the mtDNA. Only a 323 bp fragment was obtained from teeth samples from the Netherlands and the western North Atlantic (WNA); thus all populations were compared using this small fragment. Additionally, a 601 bp fragment was obtained for the UK and Norway populations (29 and 33 samples, respectively); therefore, in these populations the genetic analyses were performed comparing both fragments.

- ### Eastern North Atlantic (ENA)

From the 323 bp fragment 13 haplotypes were found among the three populations (UK, Norway and The Netherlands), defined by 13 polymorphic sites (6 singleton variable sites and 7 parsimony informative sites). Only three haplotypes were shared between these regions (Figure IV.3). Overall ($N_{Eastern}=109$) genetic diversity (Hd) (0.697 ± 0.032) was much lower than values reported for other delphinids (see discussion section) and nucleotide diversity (π) (0.0050 ± 0.0003) was similar to values that have been reported in bottlenecked populations (e.g., Weber et al. 2000). The values of both statistics for all populations are shown in Table IV.4.

When UK and Norway populations were compared using a 601 bp fragment, the number of haplotypes (defined by 21 polymorphic sites between the two regions) increased from 12 to 19. Four haplotypes were shared between the two regions, with 12 unique haplotypes restricted to Norway (Figure IV.4). The overall genetic diversity (UK plus Norway) was relatively high (0.868 ± 0.003) although these values dropped when the smaller fragment was analyzed (0.722 ± 0.043).

Table IV.3. Primers from a microsatellite library in *L. albirostris*.

Primers name	Forward 5' – 3'	Reverse 5' – 3'	Number of alleles	Annealing temperature	Mg+ Mm
Lalb15a	CCA ATT GTC CCT TCT GCC TT	AGG ACA TTG GAA CAT GCCCT	10	62	1.0
Lalb6a	GAA TCG ACT TTG AGC ATT G	CTG CTT AAC CCC ACA GAC A	10	55	1.5
Lalb3a	TGG CCT CTG AGA GTT TCT GG	TCT CTG GTT GAT GTG GTT CC	13	52	0.75
Lalb32a	AGG CTA TGT GTG TGT TTT	ATT TTG GGT GAT GAT GTG	8	59	1.5
Lalb17a*	TCA GAC CCA CCC CGA GAC	CAG CCC CCA CTA CAC ACA	-	53	0.75
Lalb28a*	ACT CTC CCT CCC AGC AAT	TCA CTC ATT AGG CAC CCC	-	50	2.5

*Only a few samples were successfully amplified in loci Lalb17a and Lalb28a.

	18	22	34	73	88	94	95	99	100	101	135	136	156	166	184	218	245	248	283	303	312	A	B	C	D
Hap 1	C	T	A	A	C	G	T	C	A	T	G	T	C	T	T	T	T	A	C	G	G	13	13	3	1
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	1	1	2	
Hap 3	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	9	5		
Hap 4	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	1			
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	14	18	19	3
Hap 6	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1		
Hap 7	.	.	.	G	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.			1	
Hap 8	.	.	.	.	.	.	G	.	.	.	.	.	G	.	C	.	.	.	.	.	.			1	
Hap 9	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	G	.	.	.			1	
Hap 10	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	C	.	.	.	.	.			1	
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.			1	
Hap 12	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.			1	
Hap 13	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.			3	2
Hap 14	A	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	A	.				1
Hap 15	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				3
Hap 16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.				1
Hap 17	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	T	A	.				1
Hap 18	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.				1

Figure IV.3. Haplotypes in western and eastern North Atlantic. **A:** UK **B**: The Netherlands **C**: Norway **D**: western North Atlantic.

Table IV.4. Genetic diversity at the mtDNA control region in *L. albirostris*.

Population	N	Number of haplotypes	Number of polymorphic sites	Haplotype diversity (H)	Nucleotide diversity (π)
323 bp fragment					
U.K	38	5	5	0.7084 ± 0.0321	0.0041 ± 0.0031
Norway	33	10	11	0.6630 ± 0.0900	0.0055 ± 0.0036
The Netherlands	38	5	5	0.6572 ± 0.0476	0.0043 ± 0.0029
WNA	13	8	12	0.9103 ± 0.0559	0.0096 ± 0.0060
601 bp fragment					
UK	29	7	8	0.8128 ± 0.0385	0.0044 ± 0.0027
Norway	33	19	19	0.8788 ± 0.0480	0.0062 ± 0.0036
Overall					
ENA 323 bp	109	13	13	0.6970 ± 0.0320	0.0050 ± 0.0003
ENA (UK-Norway) 601 bp	62	19	21	0.8680 ± 0.0033	0.0059 ± 0.0004
WNA and ENA	122	18	21	0.7320 ± 0.0031	0.0056 ± 0.0004

	22	73	88	95	99	100	136	156	166	218	245	248	329	391	392	457	458	539	542	592	599	NORWAY	UK
Hap 1	T	A	G	T	C	A	T	G	T	C	T	A	A	T	C	C	C	A	C	G	C	1	
Hap 2	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	11	9
Hap 3	.	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	2	
Hap 4	.	.	C	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.	.	1	
Hap 5	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	1	
Hap 6	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	1	
Hap 7	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	3	
Hap 8	.	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	2	
Hap 9	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	1	3
Hap 10	.	.	C	.	.	.	.	C	C	T	.	.	.	.	.	.	.	.	.	.	.	1	
Hap 11	.	.	C	.	.	.	.	C	C	T	.	.	G	.	.	.	.	.	.	.	.	1	
Hap 12	.	.	C	.	.	.	.	C	.	T	.	.	.	.	.	.	T	G	T	.	.	2	1
Hap 13	.	.	C	.	.	.	.	C	.	T	C	.	G	.	.	.	T	G	T	.	.	1	
Hap 14	.	T	C	.	.	.	.	C	.	T	.	.	.	.	.	.	T	.	T	.	.	3	
Hap 15	.	.	C	.	.	.	.	C	.	T	.	.	.	.	.	.	T	G	.	.	.	1	6
Hap 16	.	.	C	.	.	.	.	C	.	T	.	.	.	.	.	A	T	G	.	.	.	0	2
Hap 17	.	.	C	.	.	.	.	C	.	T	.	.	.	.	.	.	T	G	.	A	.	0	1
Hap 18	.	.	C	.	.	C	G	C	.	T	.	.	.	.	.	.	T	G	.	T	T	1	
Hap 19	C	.	C	.	.	.	.	C	.	T	.	.	.	.	.	.	T	G	.	.	.	0	7

Figure IV.4. Polymorphic sites and haplotypes in UK and Norway populations (mtDNA control region).

Nucleotide diversity (π) was the same for UK populations when the two fragments were compared (0.004 ± 0.003); however the values for the Norway population were slightly different (see Table IV.4).

- **Western North Atlantic (WNA)**

Although only thirteen samples were available from the western North Atlantic, the number of haplotypes in this region were fairly high (8), indicating that haplotype diversity is relatively high in this region (Hd: 0.910 ± 0.056) (Table IV.4). Nucleotide diversity was higher than in other populations (π: 0.0096 ± 0.0060). The number of haplotypes for this region and polymorphic sites are shown in Figure IV.3.

- **Western vs. eastern North Atlantic**

Nineteen haplotypes were found among western and eastern North Atlantic populations, defined by 21 polymorphic sites. Only two haplotypes were shared between these four populations (Figure IV.3). The mitochondrial genetic diversity (overall Hd: 0.7320 ± 0.0031) was moderate, in contrast with the nucleotide diversity that was low (overall 0.0056 ± 0.0004).

1.1. Differentiation among populations

An AMOVA was performed to test differentiation among groups, dividing the samples into two groups: eastern (UK, Norway and The Netherlands) and western North Atlantic. The results indicate that only 8.29% of the variance in haplotype frequencies could be explained by the differences between the western and eastern populations, while 86.25% of the variance could be explained as a result of the differences within populations (Table IV.5).

The Fst and ϕst values suggest that there is a significant differentiation between the western North Atlantic population and the three eastern North Atlantic populations, among eastern populations some degree of differentiation was found between UK and Norway and between Norway and The Netherlands, but no differentiation was found between the UK

Table IV.5. AMOVA performed to evaluate the differentiation among regional samples of *L. albirostris*.

Source of variation	df	Variance components	Variance explained (%)	P values	Fixation indices
Among groups	1	3.542	8.29	0.2552	φct: 0.0829
Among populations within groups	2	5.536	5.45	0.0086	φsc: 0.0595
Within populations	118	0.841	86.25	0.0020	φst: 0.1375
Total	121	0.975			

and The Netherlands (Table IV.6). Genetic flow values between populations from Fst and φst are reported in Table IV.7.

1.2. Phylogenetic relationships among haplotypes

Two different NJ trees were constructed in order to elucidate the relationships among haplotypes (using 601 bp and 323 bp). Both trees showed a certain degree of association between haplotypes from each region, especially those from Norway, but with very little bootstrap support (Figures IV.5a and IV.5b). The Network tree showed evidence for one or more sudden expansions of this species in the North Atlantic.

1.3. Assessing demographic parameters in L. albirostris

- ### *Demographic expansion - Mismatch distribution analysis and neutrality tests-*

No clear unimodal distributions were obtained for most of the populations analyzed (Figure IV.6), however the values of the variance (SSD) and the raggedness index (r) were small and non-significant in all populations, suggesting that the distributions did not differ significantly from those expected under a model of sudden expansion (see Roger and Harpending 1992; Schneider and Excoffier 1999; see material and methods in chapter III for an explanation about the interpretation of SSD and r). These results were not corroborated by the Tajima's D and the Fu's Fs statistics, which were positive and non-significant in most populations studied, except for Norway (Fu's Fs = -5.7633 P< 0.0095 using 601 bp (Table IV.8).

Assuming that there is no evidence to reject the hypothesis of demographic expansion in *L. albirostris*, the τ values were used to calculate the time of this expansion and the number of effective females after the expansion. The time of expansion ranged from 7506-6755 years ago (using $6.3\text{-}7.0 \times 10^{-7}$ substitutions per site per year) to 22411-15762 years ago (using a mutation rate of $2.11\text{-}3.0 \times 10^{-7}$ substitutions per site per year). Both values correspond to the highest values obtained for a population, in this case the WNA population (Table IV.9). θ_1 values are shown in Table IV.9.

Table IV.6. Fst and φst values for *L .albirostris* using the mtDNA control region.*

Region	UK	The Netherlands	Norway	WNA
UK		-0.0092	0.0918**	0.1003
The Netherlands	-0.00149		0.0498	0.1083
Norway	0.1360**	0.0605		0.0823
WNA	0.0970	0.1085	0.1445	

*Values below the diagonal are φst values and values above the diagonal are Fst values. Numbers with double asterisk (**) were calculated using 601 bp, values in bold are significant at the 0.05 level.

Table IV.7. Number of migrants per generation obtained using Fst and φst values.

Region	UK	The Netherlands	Norway	WNA
UK		infinite	2.5*	4.6
The Netherlands	infinite		7.6	4.0
Norway	10.8*	8.6		3.0
WNA	4.4	4.0	5.9	

Values below diagonal are Nm from Fst values, above diagonal are Nm from φst, figures with asterisk (*) were calculated using 601 bp fragment.
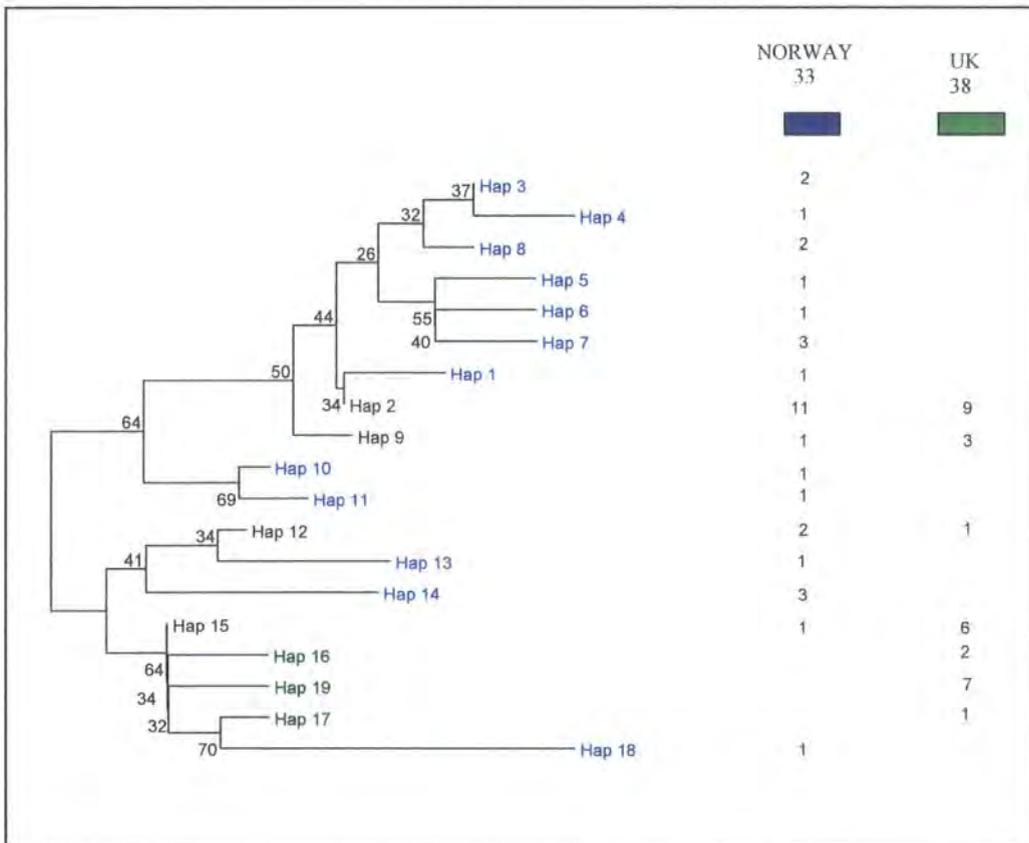
Figure III.5a. Neighbour joining tree using 601 bp. Colors in the NJ tree correspond to private haplotypes for each region.

The table within the top panel of the figure:

Haplotype	UK N=38	The Netherlands N=38	Norway N=33	WNA N=13
Hap 7				1
Hap 14				
Hap 8			1	
Hap 9			1	
Hap 5	14	18	19	3
Hap 10			1	
Hap 11			1	
Hap 6		1		
Hap 2	1	1	2	
Hap 3	9	5		
Hap 18				1
				3
Hap 15	1			
Hap 4			3	2
Hap 13			1	
Hap 12				
Hap 1	13	13	3	1
Hap 16				1
Hap 17				1
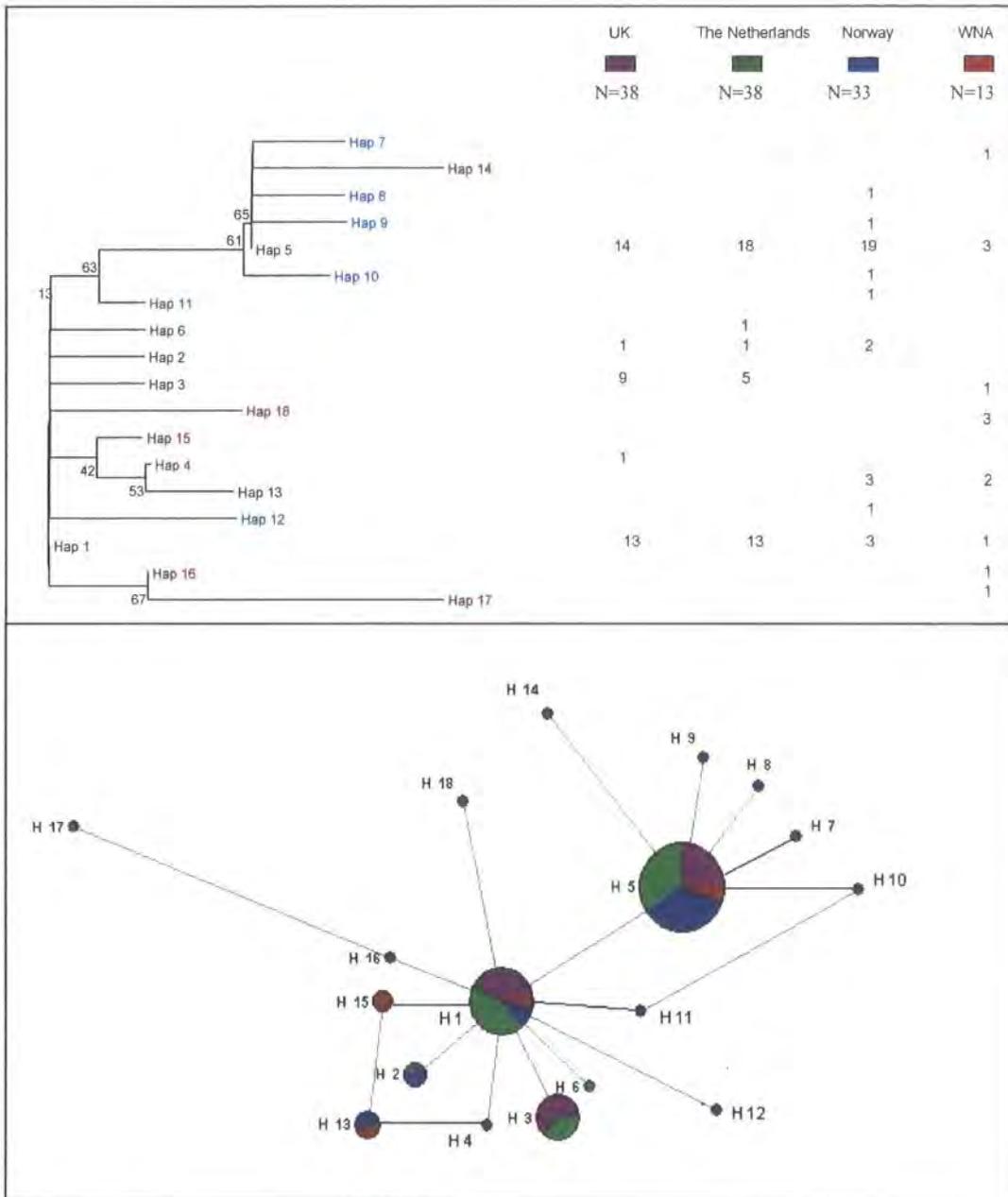
Figure IV.5b. Neighbour joining and Network tree showing the relationships among haplotypes using 323 bp. Colors in the NJ tree represent private haplotypes for each region.

*Line lengths in the network tree are proportional to the number of mutations among haplotypes.
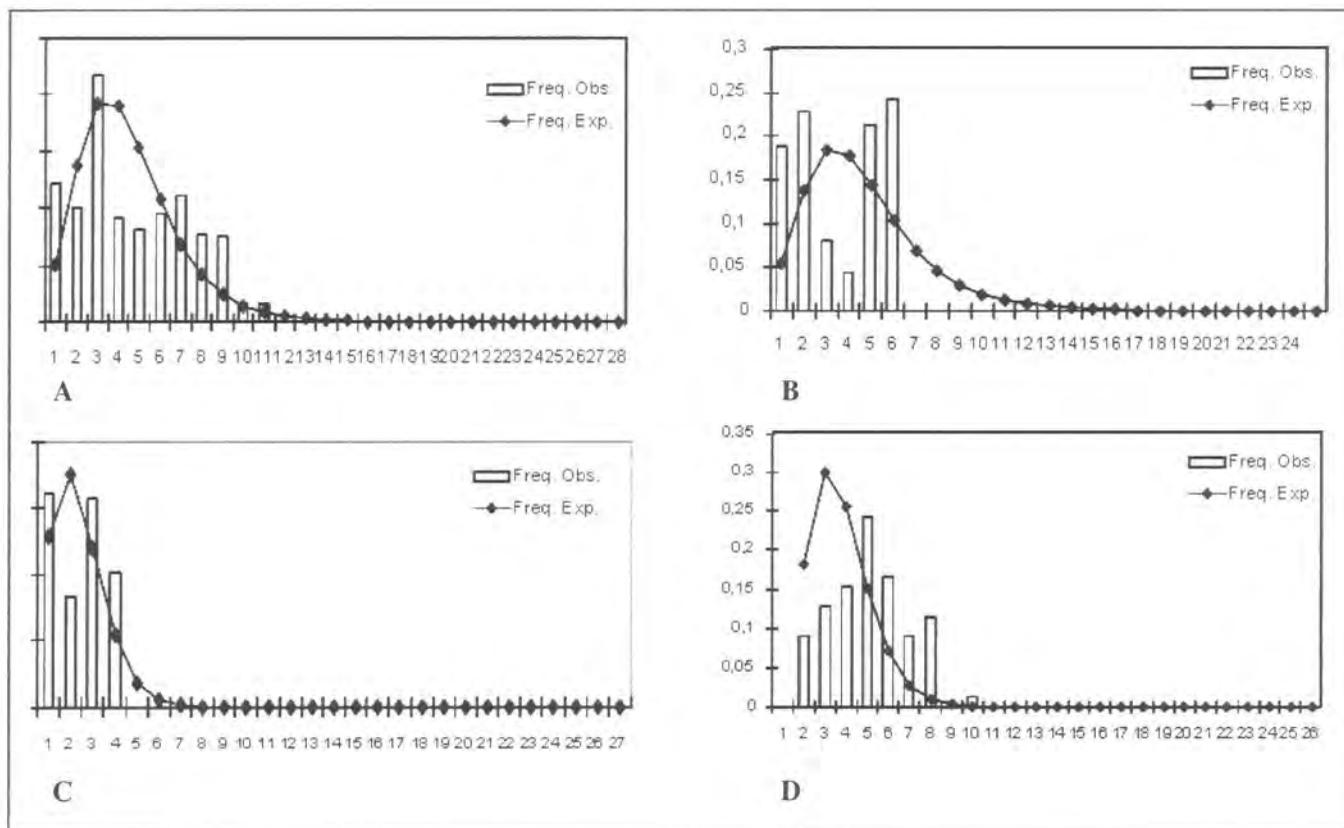
Figure IV.6. Mismatch distribution under a model of demographic expansion. The x axis shows the number of pairwise differences, the y axis shows the frequency of the pairwise comparisons. *A*. Norway population (601 bp) *B*. UK population (601 bp) *C*. The Netherlands (323 bp) *D*. WNA population (323 bp).

Table IV.8. Demographic expansion parameters.

| Statistics	UK 323 bp	UK 601 bp	Norway 323 bp	Norway 601 bp	The Netherlands	WNA
Sample size	38	29	33	33	38	13
Tau	2.4707	5.2402	0.0000	1.6582	2.6289	3.0547
θ_0	0.0018	0.0000	0.0000	3.3908	0.0000	0.5625
θ_1	3.9300	5.5594	9999*	11.0254	3.0800	15.3223
SSD	0.0140	0.0463	0.5407	0.0133	0.0412	0.0069
Model (SSD) p-value	0.3380	0.1446	0.0000	0.4755	0.1770	0.7640
Raggedness index	0.0670	0.1142	0.0521	0.0350	0.1543	0.0291
Raggedness p-value	0.6620	0.2339	1.0000	0.5284	0.2370	0.8800
Tajima's D	0.5245	0.8476	-1.1175	0.8476	0.3768	-0.8342
Tajima's D p-value	0.7356	0.8230	0.1358	0.8230	0.6880	0.2196
Fu's Fs test	0.6174	0.3254	-3.5176	-5.7633	0.4756	-1.9856
FS p-value	0.6580	0.5958	0.0276	0.0095	0.6393	0.1125

*θ Values equal to 9999 means that the population sizes is too large to be calculated with the small fragment as suggested by Excoffier (pers. comm.), therefore the value calculated from the 601 bp is more accurate.

- *Spatial expansion*

The hypothesis of spatial expansion was also tested under the infinite-island model, using the mismatch distribution. No evidence was found to reject this hypothesis in any of the populations as all raggedness indices were small and non-significant (Figure IV.7 and Table IV.10). The times of the possible range expansion are shown in Table IV.9. These values, as well as the values for the number of migrants per generation (M=Nm), changed significantly depending on the length of fragment that was used (see Table IV.10). For example, when using the longer fragment, the M value dropped 7-fold over the values calculated with the small fragment in the Norway population.

- *Under isolation-with-migration model*

Three populations were compared using the isolation-with-migration model using the program Mdiv (UK vs. Norway, WNA vs. Norway and WNA vs. UK). All parameters had the same distribution when using different chains and burn-in steps. The highest points for $\theta=2Ne\mu$ and for M=Nm (for haploid populations) are shown in Figure IV.8. The estimates for the ancestral sizes of pairs of populations and genetic flow, using the three mutation rates mentioned above are shown in Table IV.11. The T parameter did not resolve a division time for any of the population pairs analyzed as the probability for all values was the same. The time for the most recent common ancestor TMRCA was also estimated, values are shown in Table IV.11.

Table IV.9. Calculation of the expansion time ($t= \tau/2\mu$) and effective number of females after demographic expansion ($Ne=\theta_1/2\mu$).

	UK	Norway	The Netherlands	WNA
Demographic Expansion				
$t= \tau/2\mu$= years ago				
*1	**6921-6229**	**2190-1971**	6460-5814	7506-6755
2	**8720**	**2759**	8139	9457
3	**20663-14533**	**6538-4598**	19287-13565	22411-15762
$Ne=\theta_1/2\mu$.				
1	**7341-6607**	**14560-13104**	7568-6811	37649-33884
2	**9250**	**18345**	9536	47437
3	**21920-15417**	**43472-30575**	22596-15893	112411-79062
Spatial Expansion				
$t= \tau/2\mu$ =years ago				
1	**4358-3922**	**1717-1545**	5651-5086	6143-5529
2	**5491**	**2163**	7121	7740
3	**13011-9151**	**5126-3605**	16874-11868	18341-12900
$Ne=\theta_1/2\mu$				
1	**1810-2011**	**4050-4500**	1	4670-6640
3	**2534**	**5670**	2	2802
2	**4223-6005**	**9450-13436**	4-5	2001-2224

*Numbers 1,2 and 3 correspond to different mutation rates 1. Harlin et al. (2003) $\mu=6.3 - 7.0 \times 10^{-7}$; 2. Ho et al. (2007) $\mu=5.0 \times 10^{-7}$; 3. Hayano et al. (2004) $\mu=2.11-2.99\times 10^{-7}$. (Mutation rate per site per year (μ) is equal to $\lambda \times$ generation time (10 years) $\times$ number of nucleotides in this study (see Roger and Harpending 1992). Values in bold were estimated using a 601 bp fragment.
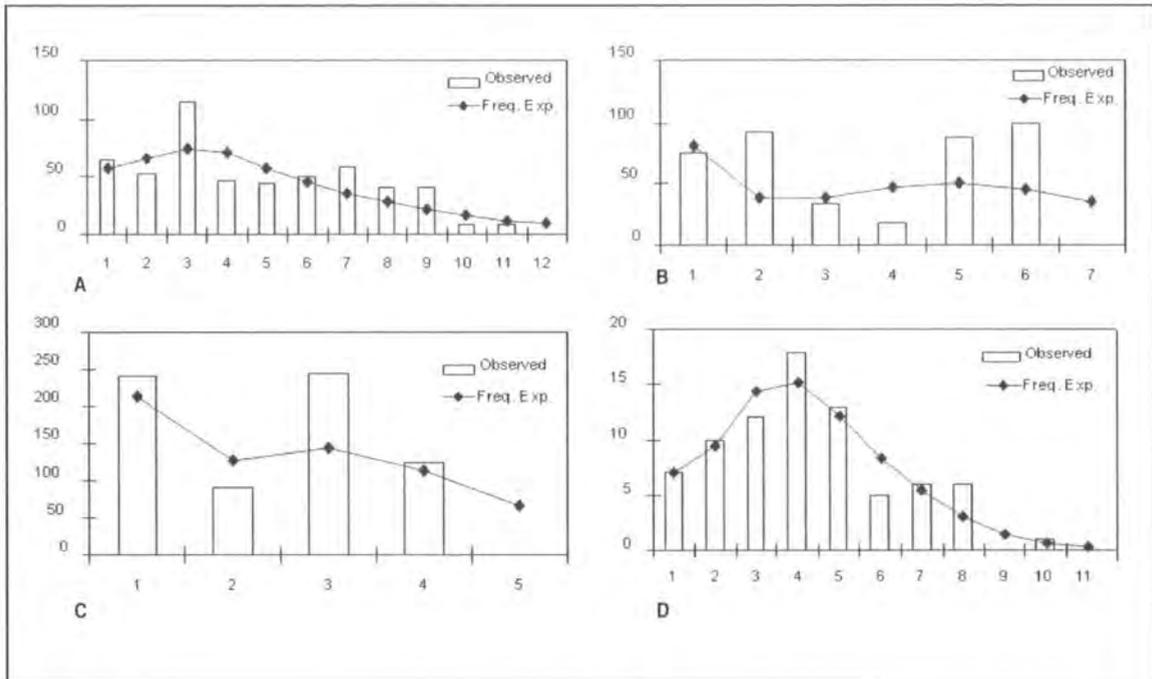
Figure IV.7. Mismatch distribution under a model of spatial expansion. The x axis shows the number of pairwise differences, the y axis shows the frequency of the pairwise comparisons. **A.** Norway population (601 bp) **B.** UK population (601 bp) **C.** The Netherlands (323 bp) **D.** WNA population (323 bp).

Table IV.10. Spatial expansion parameters in populations of *L. albirostris*.

| Statistics	UK 323 bp	UK 601 bp	Norway 323 bp	Norway 601 bp	The Netherlands	WNA
τ	2.2	3.3	2.5	1.3	2.3	2.5
θ	0.0007	1.5229	0.3885	3.4077	0.0007	0.9051
M	5.0307	2.7399	2.0588	14.714	3.3657	17.8430
SSD	0.0080	0.0500	0.0057	0.0130	0.0249	0.0065
SSD p-value	0.4690	0.0850	0.8160	0.4363	0.2520	0.7840
Raggedness index	0.0670	0.1141	0.0521	0.0350	0.1543	0.0291
Raggedness p-value	0.7030	0.5177	0.8900	0.5446	0.4490	0.8930
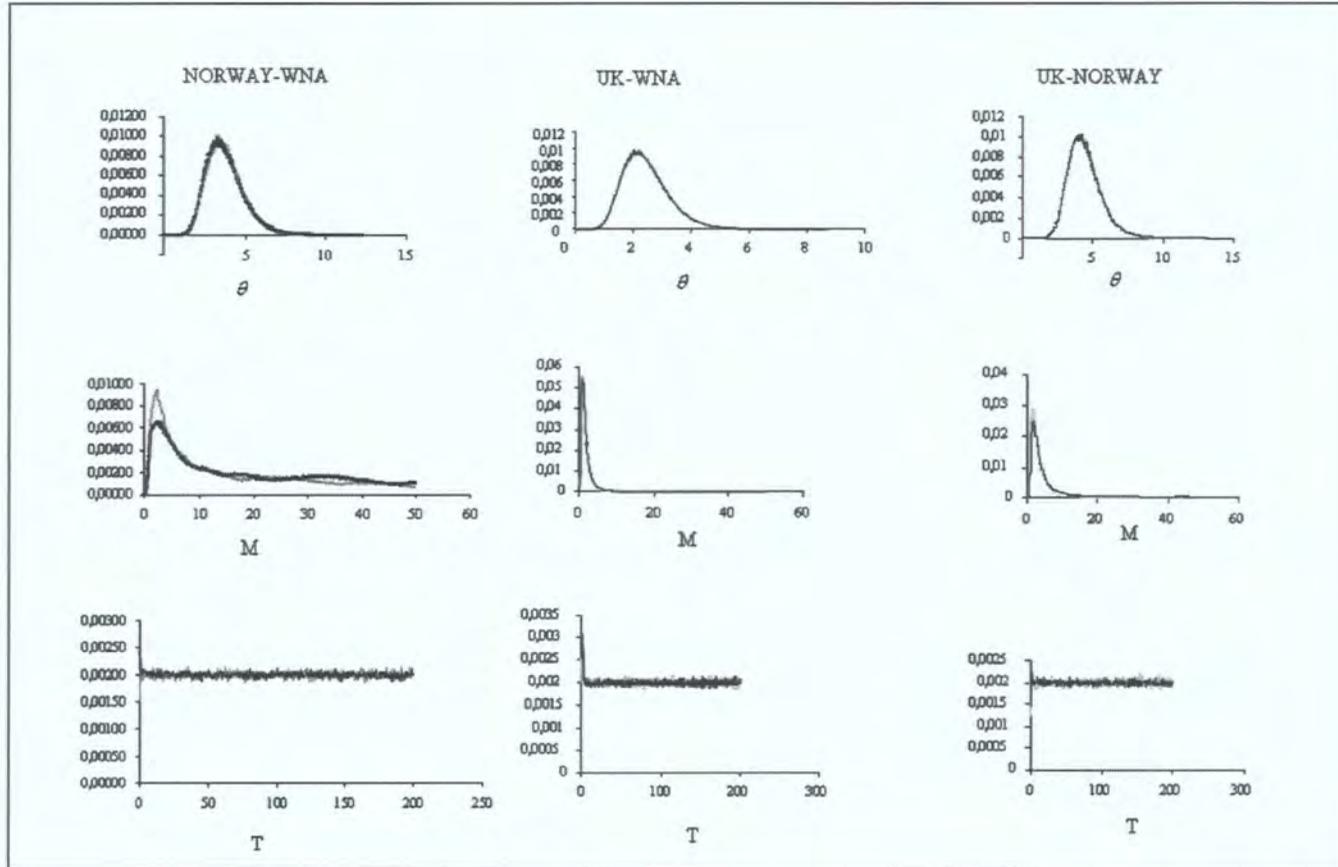
Figure IV.8. Posterior distribution of parameters θ, M and T estimated with the Mdiv program.

Note: Black lines represent the results running 10^7 chains and grey lines running 5×10^6 chains. Y axis shows probabilities.

Table IV.11. Population parameters obtained from the Mdiv program.

Population	$\theta=2Neu$	Nm	TMRCA=ut		$Ne=\theta/2u$	t
UK-WNA	2.1490	2.3	1.685	*1	5280-4752	8281-7452
				2	9288	10433
				3	15766-11089	24724-17389
Norway-WNA	3.2437	0.8	2.263	1	7970-7173	11121-10009
				2	10042	14012
				3	23797-16737	33205-23354
UK-Norway	3.9708	1.6	1.845	1	5244-4719	4873-4386
				2	6607	6140
				3	15656-11012	14549-10233

*Numbers 1, 2 and 3 correspond to different mutation rates per site per year 1. Harlin et al. (2003) μ=6.3 - 7.0 × 10^{-7}; μ=2. Ho et al. (2007) μ=5.0 × 10^{-7}; 3. Hayano et al. (2004) μ=2.11-2.99× 10^{-7}. Ne is the population size and t the time of the most recent common ancestor between the two populations. Nm was calculated from the M values ($M=Nm$).

2. Microsatellite screening and diversity

2.1. Microsatellite library

A total of 26 positive clones with a fragment above 400 bp were sequenced from the species-specific microsatellite library. Among these, 6 clones contained simple sequences of 15, 20, 54, 68, 80 and 128 di-nucleotide repeats (CA). Six sets of primers were designed (see Materials and methods section), but only four microsatellites were successfully standardized (*Lalb6a, Lalb3a, Lalb15a and Lalb32a*) and used in combination with previous standardized microsatellites to evaluate the genetic diversity of *L. albirostris*.

Samples from the UK and Norway populations were amplified with 15 loci, while the tooth and bone samples from The Netherlands were amplified with 5 microsatellites. Comparisons among the three populations were carried out using five microsatellites. No microsatellite loci were obtained for samples in the western North Atlantic.

The genotypic independence between each pair of loci was confirmed using the linkage disequilibrium test. From the 15 microsatellites evaluated in the UK and Norway populations, two loci: EV37 and EV94 showed evidence for null alleles and one locus showed evidence for errors due to stuttering, based on analysis using the program Microchecker (Van Oosterhout et al. 2004). The locus with the stutter errors (Lalb3a) was deleted from further analyses. In The Netherlands population no loci with null alleles were detected. Loci EV37 and EV94 were included in the analyses, given that all population comparison tests (Fst and Rst) performed with and without these loci showed similar results.

The genetic diversity was similar in all populations of *L. albirostris* analyzed, the lowest value being found in the Norway population (0.5453 ± 0.1415) and the highest value in The Netherlands populations (0.6732 ± 0.1022, using 5 microsatellites). The mean number of alleles was somewhat low. The allelic richness averaged across the 14 loci (UK plus Norway) was 6.01. After Bonferroni correction, implemented in the Fstat programme,

vers. 2.9.3 (Goudet 2001), two loci showed deviation from HW in the two populations analyzed UK and Norway populations (Ev37 and Ev94), and 1 locus (Di24) in The Netherlands population (Table IV.12.).

2.2. Population structure at biparental level in L. albirostris

Fst values using 14 microsatellites showed a small and significant difference between Norway and UK populations (Rst: 0.0227 P< 0.04980; Fst: 0.018 P<0.00001). Rst and Fst values showed non significant differentiation between The Netherlands and UK, and significant differentiation between Norway and The Netherlands (using five microsatellites) see Table IV.13.

2.3. Bottleneck analysis

Given that the number of loci analyzed in WNA and The Netherlands populations were small, the bottleneck analysis was performed only for the UK and Norway populations. No heterozygosity excess was found for any of the populations that were analyzed with the bottleneck program. In contrast, M-Garza values were lower than 4.0 in all populations, congruent with values expected in populations that have undergone a bottleneck.

Table IV.12. Continuation. Genetic diversity at microsatellite loci in *L. albirostris*.

Locus	Population	*N	K	R	Ho	He	*Fis	Allelic range	G-W statistics
Lalb6a	UK	26	8	7.7570	0.6154	0.7481	0.1800	32	0.2424
	Norway	44	10	8.5240	0.6818	0.7537	0.0960	22	0.4348
Lalb32a	UK	26	7	6.7570	0.7308	0.6659	-0.1000	33	0.2059
	Norway	43	8	6.5520	0.5349	0.6227	0.1420	33	0.2353
FCB4	UK	25	4	3.9200	0.7200	0.5845	-0.2380	6	0.5714
	Norway	41	6	5.3570	0.6829	0.6555	-0.0420	12	0.4615
EV37	UK	24	4	3.9990	0.3750	0.6277**	0.4080	28	0.1379
	Norway	44	10	8.9860	0.6591	0.6904	0.0460	32	0.3030
KWM2a	UK	26	6	5.7580	0.6154	0.6772	0.0930	14	0.4000
	Norway	41	4	3.5610	0.6098	0.5959	-0.0240	14	0.2667
EV94	UK	24	6	5.8740	0.2500	0.4220**	0.4130	14	0.4000
	Norway	43	7	6.1350	0.4186	0.4208	0.0050	14	0.4667
Lalb15a	UK	24	10	9.8320	0.7917	0.7642	-0.0370	22	0.4348
	Norway	31	6	5.8090	1.0000	0.6563	-0.537*	12	0.4615
Overall microsatellites (14)	UK		5.57	5.4830	0.6132	0.6210	0.0130	17.71	0.3350
	norway		6.29	5.5740	0.6233	0.5453	-0.0480	16.85	0.3772
Overall microsatellites (5)	The Netherlands		5.40	5.2600	0.6374	0.6732	0.2180	13.4	0.3968

Fis: Degree of random mating as in Weir and Cockerham (1984). N: number of samples; K: number of alleles; R: Allelic Richness; G-W: Garza-Williamson statistics; Ho: Observed heterozygosity; He: Expected Heterozigosity. Samples with deviation from HW equilibrium are denoted with two asterisks (**) and significant values of Fis are shown with one asterisk (*).

Table IV.12. Continuation. Genetic diversity at microsatellite loci in *L. albirostris*.

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Lalb32a	UK	26	7	6.7570	0.7308	0.6659	-0.1000	33	0.2059
	Norway	43	8	6.5520	0.5349	0.6227	0.1420	33	0.2353
FCB4	UK	25	4	3.9200	0.7200	0.5845	-0.2380	6	0.5714
	Norway	41	6	5.3570	0.6829	0.6555	-0.0420	12	0.4615
EV37	UK	24	4	3.9990	0.3750	0.6277**	0.4080	28	0.1379
	Norway	44	10	8.9860	0.6591	0.6904	0.0460	32	0.3030
KWM2a	UK	26	6	5.7580	0.6154	0.6772	0.0930	14	0.4000
	Norway	41	4	3.5610	0.6098	0.5959	-0.0240	14	0.2667
EV94	UK	24	6	5.8740	0.2500	0.4220**	0.4130	14	0.4000
	Norway	43	7	6.1350	0.4186	0.4208	0.0050	14	0.4667
Lalb15a	UK	24	10	9.8320	0.7917	0.7642	-0.0370	22	0.4348
	Norway	31	6	5.8090	1.0000	0.6563	-0.537*	12	0.4615
Overall microsatellites (14)	UK		5.57	5.4830	0.6132	0.6210	0.0130	17.71	0.3350
	norway		6.29	5.5740	0.6233	0.5453	-0.0480	16.85	0.3772
Overall microsatellites (5)	The Netherlands		5.40	5.2600	0.6374	0.6732	0.2180	13.4	0.3968

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Table IV.13. Fst and Rst values for microsatellite data 10000 permutations.*

Region	UK	The Netherlands	Norway
UK		0.0106	0.0227 (14 loci)
The Netherlands	0.0041		0.0139 (5 loci)
Norway	0.0185	0.0285	

*Values above the diagonal are Rst values and below the diagonal are Fst values. Norway vs UK were compared using 14 microsatellites. Netherlands was compared against the other populations using five microsatellites.

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DISCUSSION

1. Genetic Diversity in Lagenorhynchus albirostris

The diversity at the nuclear level in *L. albirostris* was moderate (ranging from 0.5453 ± 0.1415 to 0.6732 ± 0.1022) and similar to values reported for other delphinids populations (e.g., Hayano et al. 2004; Buchannan et al. 1996). The mitochondrial genetic diversity (overall Hd: 0.7320 ± 0.0031) was also within the range described for other cetacean species (from 0.68-0.96) (e.g., Querouil et al. 2007; Natoli et al. 2006; Hayano et al. 2004; Cassens et al. 2003; Harlin et al. 2003; Pichler and Baker 2000). In contrast, the nucleotide diversity was very low (ranging from 0.0043 in The Netherlands to 0.0096 in the WNA population) and similar only to values reported in those cetacean populations with historically small population sizes or that have been strongly affected by human activities (Natoli et al. 2006; Bérubé et al. 1998).

The low nucleotide diversity (overall 0.0056 ± 0.0004) found in *L. albirostris* together with a moderate haplotypic diversity supports the hypothesis that this species has undergone one or several bottlenecks, which may be related to its limited distribution to coastal areas and the effects of Pleistocene glaciations on the diversity of temperate species (see Wares 2002; Hewitt 2000, 2004). This hypothesis was corroborated by the analysis of the distribution of pairwise differences (mismatch distribution) which is discussed below.

1.1. Testing historical bottlenecks and a sudden demographic and/or spatial expansion in L. albirostris: Evidence from the mismatch distribution and neutrality tests

After a catastrophic decline in size, it is likely that many species present a sustained increase in population size, showing a model of exponential growth at a constant rate (Slatkin and Hudson 1991). This expansion leaves recognizable signatures in the pattern of molecular diversity in species; for example spatial expansion and sudden demographic expansion lead to unimodal distributions of pairwise differences more than to a geometric

distribution expected in populations that have remained constant in size (Schneider and Excoffier 1999; Roger and Harpending 1992).

The results of this study suggest a sudden demographic expansion followed by a spatial expansion in all the regional populations of *L. albirostris* that were studied. Although the demographic expansion was not corroborated by the Tajima D test and was supported by *Fu's* test only in the Norway population, not enough evidence was found to reject the hypothesis of sudden expansion in all populations.

The fact that just a few haplotypes are common among distant populations, and the star-like shape of the network, suggest the possibility of one historical expansion event (see Figure IV.5), predating population subdivision in this species. Using the mutation rates for the control region calculated by Harlin et al. (2003) for *Lagenorhynchus obliquidens* (μ= 6.3 - 7.0 $\times$ 10^{-7}) and a average of the mutation rates reviewed by Ho et al. (2007), the demographic expansions for most populations of *L. albirostris* (UK-The Netherlands and WNA) probably occurred after the Last glacial maxima, ranging between ~9457 and ~5814 years ago (see Table IV.9). Spatial expansion probably occurred several thousands of years after the demographic expansion (~7740- 3922 years ago). In contrast, more recent sudden demographic and spatial expansions were detected in the Norwegian population (~2759-1971 years ago and ~2163-1545 years ago, respectively), showing that the diversity of *L. albirostris* populations could have been shaped by different waves of spatial expansion. According to Excoffier (2004), species could have experienced range expansion following climatic change, while a local demographic expansion may be due to some local adaptations (see discussion in Excoffier 2004).

A sudden demographic expansion, together with a spatial expansion as found in *L. albirostris*, has also been detected in some other coastal marine species, especially fish (e.g., Adams et al. 2006; Costedoat et al. 2006; Haney et al. 2007; Gysels et al 2004; Wares 2002). This has been attributed to climatic changes that took place during the Pleistocene Era, which generated several episodes of range expansions and contractions, with subsequent fluctuations in population sizes in several species (Hewitt 2000; Taberlet

et al. 1998). During glaciated epochs changes in the sea level, temperatures, upwelling patterns and prey distribution seem to have played an important role in the connection and isolation of populations (e.g., Harlin-Cognato et al. 2007; Costedoat et al. 2006; Cassens et al. 2003). Therefore, it is possible that this led to a dynamic pattern of population colonisation and expansion, and a complex genetic signal of population founding and recovery in *L. albirostris*.

The effective population sizes (*Ne*) after the spatial expansion in the three populations were smaller than the values found after the demographic expansion (see Table IV.9). These results can be an indication of the presence of other past processes affecting and reducing the population sizes of these species; i.e. isolation of demes during spatial expansion (see Excoffier et al. 2004 and Wegmann et al. 2006).

- *Evolutionary rates and their impact on dating events*

The assumption that the rate of nucleotide substitution is constant, allowing inference about divergence times among species, has been controversial since the neutral theory of the molecular evolution arose (Kimura 1983). However, it is known that the nucleotide substitution rate can vary among lineages (e.g., Rooney et al. 2001) and therefore influence the calibration of the molecular clock.

The evolutionary rate proposed by Hayano et al. (2004) ($\mu = 2.11\text{-}2.99 \times 10^{-7}$) seems to over estimate the values, however the evolutionary rates proposed by Harlin et al. (2003) ($\mu = 6.3\text{-}7.0 \times 10^{-7}$ and Ho et al. (2007) ($\mu = 5.0 \times 10^{-7}$) gave estimates that were more in agreement with dates expected for post Pleistocene expansions. However, all the estimates presented in this study should be interpreted with caution.

1.2. Ancient vs. recent bottleneck in L. albirostris: testing a recent reduction in effective population size with microsatellite data

In order to address whether the populations of *L. albirostris* have experienced further reduction in population sizes due to a recent bottleneck, the program Bottleneck and the M

value proposed by Garza and Williamson (2001) were analyzed. The former tests the hypothesis that the population is in mutation-drift equilibrium, using several statistical methods for assessing the significance of any heterozygote excess, under three mutation models: SMM, TPM and IAM. Garza's M uses microsatellite data under the SMM model to test the relationships between quantitative and spatial diversity to identify recent reductions in population size (see Garza and Williamson 2001 and methods section in Chapter III).

The results of Bottleneck showed that UK and Norway populations of *L. albirostris* are not in mutation-drift equilibrium (11/14 and 13/14 loci with heterozygosity deficiency), but probably not due to a recent bottleneck (where excess heterozygosity would be expected), but possibly to a long period of size increase without immigration (see Cornuet and Luikart 1996). In contrast, values using Garza's M were low and congruent with values expected in populations that have undergone a bottleneck (≤ 4.0).

Given that both tests look over a different time frame, caution should be taken when analyzing the data, especially those drawn from the sign test: first because some of the loci used presented a departure from HW equilibrium, which can bias the results obtained (Cornuet and Luikart. 1996); and second, in agreement with the authors, the programme can detect a recent bottleneck better when a minimum of 20 microsatellite loci are studied (in this study only 14 microsatellites were evaluated). Thus further analyses are required before concluding that the population sizes in this species have not been affected recently.

2. Phylogeography, population structure and demographic history of L. albirostris

In this study evidence was found at the mtDNA level to suggest that *L. albirostris* is divided into three populations in the North Atlantic: one population in the NW Atlantic, one continuous population around Scotland (UK) and North of Europe, and one isolated population in the coastal waters of Norway. These results are in agreement with previous craniometrical analyses published by Mikkelsen and Lund (1994), who found two separate populations of *L. albirostris* in the North Atlantic.

- *Norway vs. UK populations*

The Fst and φst statistics from mitochondrial data (Fst: 0.0918 P<0.0050 φst: 0.1360 P<0.0023) and the Rst and Fst statistics for microsatellite data (Rst: 0.0228 P< 0.0498; Fst: 0.0185 P<0.0004) found in Norway and the UK populations of *L. albirostris* suggest a significant differentiation between these two populations, similar to values for microsatellite data found between different coastal populations of *L. obscurus* (.i.e. South Africa vs. Argentina; Cassens et al. 2005), populations of *Phocoena spinipinnis* within Peruvian waters (Rosa et al. 2005) and to mitochondrial estimates in populations of bottlenose whales (*Hyperoodon ampullatus*) between the eastern (Iceland) and western North Atlantic (Dalebout et al. 2006).

The fact that both statistics (φst, based on both genetic distances and haplotype frequencies; and Fst, based only on haplotype frequencies) detected genetic differentiation in these populations indicates that this differentiation may have taken place long ago as φst can detect population structure only when there has been sufficient evolutionary time for genetic divergences to evolve (O'Corry-Crowe et al. 1997). This finding was also supported by the migration-with-isolation analysis, that suggested unidirectional migration (from UK to Norway) and ocasional past exchange of migrants between the two populations (past M was ~2 females every three generations) The consistent genetic differences shown by the two markers in both populations (control region and microsatellites) also suggest that the differences between these populations are not due to differential genetic flow mediated by females or males as suggested for a great number of delphinids (e.g., Cassens et al. 2005; Natoli et al. 2005; Hoelzel et al. 2002).

On the other hand, it is recognized that the north-eastern region of the eastern North Atlantic is characterized by a shallow area with the slope of the Continental Shelf running outside the North Sea, followed by a significantly deeper western area (Mikkelsen and Lund 1994). Thus it is possible that the division between the Norway and UK populations caused by climatic changes in the past has been maintained by the existence of this natural

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barrier between the two populations, especially taking into account the low range of dispersion of this species and its restricted distribution in shallow waters.

The time of isolation between these two populations could not be established using coalescence analysis under the isolation-with-migration model; however a rough estimate of divergence time (~8000-8900 years) was obtained calculating the nucleotide divergence between the two populations (Da: 0.00112) and the relations λ= Da/2T (λ=6.3 - 7.0 × 10^{-8}; Harlin et al. 2003). This estimate was close to the estimate for the demographic expansion in UK (~6000-7000 years ago).

- ***Western North Atlantic vs. eastern North Atlantic populations***

The data in this study also support the differentiation between the western and eastern populations; but as no microsatellite data were available for this region, this differentiation was assessed only at the mitochondrial level.

Among the pairs of populations, Norway and WNA had the highest level of differentiation (0.1445 P< 0.029). The time of splitting between these two populations, calculated using the Da divergence values (Da: 0.00102), was estimated at 7285-8095 years ago, this time being older than that estimated between the UK and WNA populations (Da: 0.00049; T: 3500-3900 years ago), and similar the divergence between UK and Norway (~8000-8900 years ago) suggesting that the Norway population was possibly the first to diverge.

The isolation with migration analysis suggested that the ancestral migration between Norway vs. WNA populations (~4 females every 5 generations) was lower than ancestral migration between UK vs. WNA (~2 females per generation). However, given that the existence of intermediate populations between eastern and western North Atlantic can not be denied, this data should be interpreted with caution.

2.1. Western –eastern North Atlantic colonization in L. albirostris?

A clear picture of the route of dispersion and migrational process in this species could not be fully addressed in this study, primarily for the lack of sampling in continuous regions. The data obtained thus far can give only a rough idea and establish a preliminary hypothesis about a possible dispersion route of this species from the western North Atlantic towards the eastern North Atlantic, which should be tested with further analyses and a better representation of the populations existing in this species in the North Atlantic (i.e. Greenland, Iceland, the Faroe Islands and northern and south-western North Atlantic).

The western North Atlantic and the eastern North Atlantic are connected by Greenland, Iceland and the Faroe Islands and separated by the possible barrier effect of the deeper waters of the southern part of the mid-Atlantic (Mikkelsen and Lund 1994). Thus it is possible that the colonization of *L. albirostris* took place from the western toward the eastern North Atlantic using the marine bridge between these areas before the decline in sea levels during the Pleistocene Epoch (see Mitrovica 2003; Bigg et al. 2008). This premise can be supported as the western North Atlantic population of *L. albirostris* analyzed in this study had the highest effective population numbers and the highest nucleotide diversity despite the limited number of samples (13) and it has been recognized that populations with the highest nucleotide diversity frequently are the central range of dispersion for a given species. Unfortunately, these results could be biased by the small samples size of the western north Atlantic population, and therefore this hypothesis should be tested with further studies and a good sampling representation of this region.

Given that the Norway population seems to diverge early in time (~8000-8900 years ago), it is possible that a few individuals travelled further North and East, reaching the North of Norway during the Pleistocene epoch, becoming isolated from the other populations as suggested by the number of private haplotypes in each region (especially in Norway), the low number of shared haplotypes between WNA and Norway (3 out of 19), and the low nucleotide diversity in this population.

Furthermore, the individuals that colonized UK waters could have probably approached from the western North Atlantic rather than those that had been established previously in the northern North Sea (i.e. Norway) via Iceland as the φst values showed greater differentiation between the UK and Norway than between the UK and WNA samples. This is, however, speculative because the existence of intermediate population between the two regions has not been determined. This discussion could be enriched in the future with further population analyses throughout the geographic range of the species.

CHAPTER V: SYNTHESIS

AN APPROACH TO THE EVOLUTIONARY AND DEMOGRAPHIC
HISTORY OF THE CURRENT GENUS *LAGENORHYNCHUS*
AND A COMPARATIVE PHYLOGEOGRAPHY OF
L. ACUTUS AND *L. ALBIROSTRIS*

1. An approach to the evolutionary and demographic history of the current genus
Lagenorhynchus

This study showed the usefulness of combining nuclear and mitochondrial genes to define the relationships amongst the species of the genus *Lagenorhynchus*. The nine genes used in this study fully resolved the relationship amongst the species of this genus and provided new insights into the phylogeny of the family Delphinidae. Moreover, the independent analysis of each gene shows that although individual nuclear and mitochondrial genes can give a preliminary understanding about the relationships between species, they do not always recover the same relationships and can generate markedly different phylogenetic hypotheses. Therefore combining different data sets in a total evidence phylogeny seems to be the best approach for recovering relationships between closely related species as suggested by other authors (e.g., Gatesy et al. 1999; Baker and Desalle 1997; DeSalle and Brower 1997; Huelsenbeck et al. 1996).

This study has provided several new insights towards understanding the phylogeny of Delphinidae: First the resolution of the basal position of *L. acutus* and *L. albirostris* in the phylogeny of delphinids, with *L. acutus* appearing as the basal species of Delphinidae, and providing the first molecular support to include this species in a new subfamily. Second the identification of non-monophyletic relationships between *L. acutus* and *L. albirostris*, suggesting that these two species should probably be placed in different genera. This suggestion was previously addressed by LeDuc et al. (1999); however their phylogenetic analyses did not resolve the relationships between these two species and the other delphinids. Third the placement of *L. cruciger* and *L. australis* as close related with the genus *Cephalorhynchus*.

The outcome of this research disagrees to some extent with the hypothesis that the species of the genus *Lagenorhynchus* distributed in the North Hemisphere originated as a consequence of the transgression of the tropical barrier during the Plio-Pleistocene glacial periods, as proposed by early studies about antitropically distributed species (see Cipriano 1997, Davis 1983). Therefore, in this study a new hypothesis about the dispersion and speciation of the species currently classified in the genus *Lagenorhynchus*

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is proposed, based mainly on a combined interpretation of several points: First, the phylogenetic relationships amongst the species of the genus showed by the total evidence tree (see Figure II.16), the splitting time between *L. acutus* and *L. albirostris* (~6.0 MY ago), and the time of the most recent common ancestor between both species (~6.53 MY ago). Second, the changes in the marine environment during the Miocene-early Pliocene proposed by several authors and their effects on marine organisms evolution (e.g., Berger 2007; Raymo et al. 2006; Matul and Abelmann 2005; Briggs 2003; Raymo 1994), and third, previous studies about the radiation and speciation of *L. obliquidens-L.obscurus* in the Pacific Ocean (Harlin-Cognato et al. 2007; Hare et al. 2002).

As fully explained in chapter II in the aforementioned hypothesis, I suggest that the ancestral species of *Lagenorhynchus* probably appeared in the North Atlantic Ocean basin around the Miocene-early Pliocene epoch and migrated either toward the North Pacific, probably via the Bering Strait; or migrated southwards in the Atlantic Ocean, followed by an incursion in the Pacific Basin before the closure of the Panamic portal (~3.1 MY ago), probably guided by the establishment of new upwelling regions in subtropical areas (see Berger 2007). Populations that remained in the Atlantic Ocean probably migrated North during the cooling of the oceans, when the North hemisphere become rich in upwelling areas and the availability of feeding grounds was higher than in subtropical regions (Berger 2007), thus *L. acutus* and *L. albirostris* begun to diverge and evolve in different ecological niches (see next section); it is probable that the populations that migrated early toward the North Pacific Ocean diverged from North Atlantic populations by vicariant speciation, as a result of the intermittent closure of the Bering Strait during glaciated epochs or due to the closure of the Panamic portal in the middle Miocene. As suggested by Harlin-Cognato et al. (2007) and Hare et al. (2002), populations of *L. obliquidens* probably travelled North to South in a dispersion event, giving origin to *L. obscurus*. The transgression of the tropical barrier was possibly due to the high availability and broad distribution of prey in the tropical seas (Harlin-Cognato et al. 2007).

The next step in this research should be to integrate a major number of species of the family Delphinidae and other odontocetes in a multilocus analysis. For example, the

inclusion of more cetacean species, including the four *Cephalorhynchus* species and at least two out-groups would allow a better resolution of the classification of the family Delphinidae and a better understanding of the ancestor-descendant polarity. Moreover a better calibration of the molecular clock will be necessary to give further support to the hypothesis suggested in this study.

2. *Comparative phylogeography and population structure of L. acutus and L. albirostris in the North Atlantic*

The climate changes during the Pliocene (~5.3-1.8 MY ago) and the Pleistocene (~1.8-0.01 MY ago) epochs, especially the Pliocene cooling event, starting ~2.4-2.9 MY ago and the changes over the last 0.7 MY, finishing with the last glacial maximum (LGM, ~0.21 MY ago), have been recognized as the principal factors leading to the speciation, diversification and contemporary distribution of organisms in the northern hemisphere (e.g., Chevolot et al. 2007; Carstens and Knowles 2007; Matul and Abelmann 2005; Waltari et al. 2004; Hewitt 2004, 2000, 1996; Briggs 2003; Wares and Cunningham 2001). This is especially true in the North Atlantic, where several marine species have shown patterns of genetic diversity consistent with a limited range of distribution and with the posterior expansion during interglacial periods, possibly due to changes in the ice sheet covers, changes in ocean current patterns and fluctuation in sea temperatures during glaciated epochs (e.g., Pastene et al. 2007; Addison and Hart 2005; Bigg et al. 2008; Chevolot et al. 2007, Vermeij 2005).

In this study the phylogeographic history of two endemic species of dolphins distributed in the North Atlantic (*L. acutus* and *L. albirostris*) and the possible effect of glaciation events on the genetic diversity, distribution and diversification of their population was tested using coalescence analyses and the distribution of pairwise differences, together with the analysis of neutrality tests (Tajima D and Fu's Fs), which are susceptible to expansion signatures.

The results in this study showed that the ancestral lineage of *L. acutus* and *L. albirostris* was present in the North Atlantic a long time ago (~6.53 MY ago) and that both species

started to diverge approximately ~6.0 MY ago. During their evolution both species have developed differences in habitat use and temperature preferences; for example *L. acutus* is mainly found in waters with temperatures >12°C whilst *L. albirostris* prefers colder waters <12°C. Both have different patterns in the use of habitat. *L. albirostris* is mainly restricted to coastal and shallow waters <120 m depth, whilst *L. acutus* is mainly found in deep waters ~400-500 m (Das et al. 2003; Hamazaki 2002; Weinrich et al. 2001; Reeves et al. 1999; Evans 1992; Gaskin 1992; Mikkelsen and Lund 1994). Moreover, although both species are frequently seen in temporary aggregation, it is unlikely that this aggregation is related to feeding behaviour, given that the feeding habitats of these species do not overlap (MacLeod et al. 2007; Das et al. 2003).

The partial sympatric distribution of these species is not unusual, as stated by Marko (2005, p. 561): "For marine organisms, secondary contact following allopatric speciation may be a common phenomenon because oceanographic barriers that are responsible for initiating speciation are often transitory in nature and as a consequence, many recently diverged sibling species have completely or partially overlapping geographic distributions in the marine environment" (see also Palumbi 1994 and Natoli et al. 2005).

A close analysis of the forces that have driven the evolutionary history of *L. acutus* and *L. albirostris* in the North Atlantic has shown that the last Pleistocene glacial maxima had a strong effect on the genetic variability of these species and on the history of their populations. For example, both species showed the pattern of moderate haplotypic diversity/low nucleotide diversity, as expected in populations that have undergone one or several bottlenecks in their past history (Hd: $0.9170/\pi:0.0095$ in *L. acutus* and Hd: $0.697/\pi:0.00496$ in *L. albirostris*), followed by waves of demographic expansion that normally cause the presence of many haplotypes with few differences at the nucleotide level (Qu et al. 2005; Roger and Harpending 1992; Slatkin and Hudson 1991). The time of a possible expansion was similar in populations of both species, going back to the early interglacial period (i.e. WNA_{acutus} 8022-7220 year ago; $WNA_{albirostris}$ 7506-6755 years ago).

Interestingly, the expansion signal was more evident in *L. acutus* than in *L. albirostris*, and the number of effective females after the expansion was approximately 10 times higher in the former species (i.e. WNA_{acutus}: ~378000-270000 individuals and $WNA_{albirostris}$: ~9200-6600 individuals). This outcome can be explained as a result of the differences in habitat for the two species. As explained above, it would be expected that individuals of *L. acutus* can avoid the limitation of habitat by expanding to new places (i.e. migrating south of their range); whilst *L. albirostris*, which has limited dispersion capacities and is restricted to coastal areas, could have been shifted into refugial coastal marine zones with the subsequent decline in population sizes and loss of genetic diversity due to genetic drift acting in small populations. This last premise is also supported by the finding that *L. albirostris* populations fit better to a model of spatial expansion than to a model of demographic expansion (see chapter IV). Some potential refugial zones have been proposed for marine fauna, one about the latitude of Newfoundland (47°N) in the western Atlantic and Northern Portugal/Spain (43°N) in the eastern Atlantic (Chevolot et al. 2007). Palaeoclimatic and genetic evidence also show that southern Iceland could have acted as a refugial zone as well for coastal distributed species (Bingham et al. 2003; Wares and Cunningham 2001; Addison and Hart 2005). As discussed by Hewitt (2004), some temperate species followed a rapid form of expansion and some not. The expansion will depend on particular conditions; for example the barriers to dispersion, habitat distribution, and the localization of the founder populations.

Studies on other marine species have also shown that coastal species were strongly affected by the conditions during the LGM. In the North Atlantic, for instance, Bigg et al. (2008) stated that the environment during this period became especially unfavourable for species occupying polar and sub-polar shallow water habitats. Most of the sea shelf habitats were lost with the advance of the ice sheet over the continental shelf, and the global sea level fell by 120–135 m (Mitrovica 2003; Bigg et al. 2008). "This habitat loss was particularly acute along the western and northern shores of the North Atlantic, where the continental shelf is narrow." Bigg et al. (2008 p. 163).

In addition, differences in the population composition of both species were also found in this study. While *L. acutus* could be considered as composed of one continuous

population throughout its geographic range, *L. albirostris* populations showed clear differences between the western and eastern North Atlantic. Once again, this is not surprising given that the great dispersal capacities in *L. acutus*, which are supported by the recognized offshore habitat of these species and the observation of large aggregation of individuals in the mid-Atlantic (e.g., Evans and Hammond 2004; Evans 1991; Selzer and Payne 1988), could prevent differentiation between populations due to high gene flow across the North Atlantic as found in other marine organisms with high dispersal capacities (e.g., Bremer et al. 2005; Ely et al. 2005; Avise 2000). In contrast, the restrictions of *L. albirostris* to coastal habitats can generate the differentiation between populations promoted by the barrier between the western and eastern Atlantic and the deep waters in the western area in the eastern North Atlantic –i.e. Northern Norwegian regions and Great Britain (Mikkelsen and Lund 1994).

Finally, this study also showed that *L. acutus* and *L. albirostris* follow the general pattern of western-eastern dispersion found in other species (see review in Briggs 2003). In agreement with Briggs (2003), the western Atlantic has been claimed as the principal centre of dispersion of marine species, given that many species have evolved in the western Atlantic and have been capable of migrating successfully across the mid-Atlantic barrier. This is applicable to *L. acutus*, in which the deep waters of the Atlantic do not seem to act as an effective barrier for dispersion. However dispersion of *L. albirostris* from the western to eastern North Atlantic probably followed the path of the North Greenland-Iceland-Faroe Islands, which form a continuous connection between both regions (Mikkelsen and Lund 1994).

In conclusion, it is evident that the Pleistocene events have different effects on the phylogeography and population structure of *L. acutus* and *L. albirostris*, clearly correlated with differences in habitat use and dispersion capacities of both species.

3. Conservation implications and recommendation for future studies

This study is the first in the literature to present data about genetic variability at the mitochondrial and nuclear levels of *L. albirostris* and *L. acutus*. The results show that past demographic events have generated a strong reduction in population sizes in all the populations studied. Although a further reduction due to anthropogenic factors could not be established with the results obtained, it is important to recommend special plans for protecting populations in these species and their habitats. Of the two species, *L. albirostris* perhaps requires more attention for conservation plans: First because all its populations had very low genetic and nucleotide diversity (lower than *L. acutus* populations) and second, because its restriction to near-shore habitats can make it more vulnerable to human activities in coastal regions.

Special attention is also recommended for the Norwegian population of *L. albirostris* and The Netherlands population of *L. acutus*. Both populations are important targets for conservation, given that they are clearly different from the rest of the populations in their respective species. Populations that are isolated and with low effective population sizes are more likely to be affected by genetic drift than populations with large population sizes, and are therefore more susceptible to changes in habitat, bycatch and pollution.

In order to increase the impact of this work, further analyses are required in several populations of *L. albirostris* and *L. acutus*. For example sampling populations of *L. albirostris* along the coast of the United States, Canada, Iceland, Faroe Islands and Norway, as well as along the Northern and Southern North Sea will allow a better understanding about the current and past dispersion and migrational events in this species. Moreover, given that the Norwegian population seems to be isolated from the other eastern populations, a better sampling along the Norwegian coast will provide a better picture of the processes affecting these populations and leading to their diversification in the furthermost distribution range of the species.

The sampling of *L. acutus* populations along the US coast will help understand whether or not the differentiation found in this study between the ancient and modern populations

is due to changes in the populations during recent generations or is instead a clear reflection of subdivision between populations in the western North Atlantic. Better sampling will also corroborate whether or not the population stocks proposed by Palka et al. (1997) are supported by genetic data.

On the other hand, most samples for this study came from museum material (i.e. teeth and bones) from the Netherlands, the UK and the western North Atlantic. Although museum material is frequently a good resource for genetic studies, there were several problems associated with the extraction of good-quality DNA from these samples; therefore it would be worthwhile to recommend an extra effort to collect samples from stranded animals or biopsies from living animals that will provide better sample availability for future molecular studies in both species.

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