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Molecular Ecology of Bottlenose (*Tursiops* sp.) and Common (*Delphinus* sp.) Dolphins

by

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University of Durham

2004

This thesis is submitted in candidature for the degree of

Doctor of Philosophy

ABSTRACT

Bottlenose (*Tursiops* sp.) and common dolphins (*Delphinus* sp.) are amongst the most common small cetaceans. They both have a world-wide distribution from warm temperate to tropical waters inhabiting pelagic and coastal waters, and they both show high morphological variability throughout their range, leaving the taxonomic issues in both species unresolved.

This thesis provides a comparative assessment of these two species, by molecular analysis, especially in the context of comparing coastal and pelagic populations of each species. The aim is to better understand the evolutionary processes and the factors involved in shaping the population structure in small delphinids. The strategy I followed was: 1) Analyse the population structure of the bottlenose and common dolphins on a worldwide scale and compare large scale patterns in the context of known similarities and differences with respect to life history. 2) Analyse populations on a smaller geographic scale (Mediterranean Sea and South Africa) to further understand the relationship between habitat and population genetic structure.

On a worldwide scale, bottlenose dolphins showed high genetic diversity and strong population structure, both between different and similar morphotypes, suggesting limited gene flow. Two populations, of the same morphotype, have diverged considerably to the extent that they should be considered different species. Common dolphins showed lower genetic diversity and weak population structure even over a large geographic range, suggesting higher level of gene flow. However, this species also has similar morphotypes that were genetically differentiated from one another. On a smaller geographic scale, we found a similar pattern of population structure, with the bottlenose dolphin showing higher population divergence than common dolphins. However, both species provided evidence supporting the role of habitat in defining population structure in these species. These findings should facilitate the development of effective conservation and management strategies for these species, especially for the specific case studies for populations in the Mediterranean Sea and off the Natal coast of South Africa.

To the past and the next generations

To my parents and my son

ACKNOWLEDGEMENTS

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.

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PREFACE

Marine mammals are one of the best examples of ability of mammals to adapt to different environments. Over the last 60 millions years (the estimated time since mammalian ancestors first re-colonised the oceans) they have totally adapted to aquatic life reshaping their body, reorganising their biological functions, specialising in different diets, and developing complex social structures and breeding strategies. The role of the environment in this evolutionary process and the mechanisms that have governed this process in the marine environment are still subject of research in many fields of biology.

My study provides a comparative molecular analysis of two genera of small cetaceans: the bottlenose dolphin (*Tursiops sp.*) and the common dolphin (*Delphinus sp.*). Bottlenose and common dolphins are closed related and both belong to the family Delphinidae, subfamily Delphininae. They are relatively young taxa originating in the early Pliocene, around 5 million years ago (Barnes, 1990).

I applied genetic methods to investigate the evolution of the population genetic structure of these two taxa. By comparing two similar taxa I aim to draw conclusions about general mechanisms that play a role in shaping population structure in small cetaceans and which may ultimately drive the evolution of delphinid species. Understanding these mechanisms can provide useful information for the formulation of effective conservation strategies.

The strategies I have undertaken to address this question are:
1. Analyse the population structure of the bottlenose and common dolphins on a worldwide scale and compare large scale patterns in the context of known similarities and differences with respect to life history
2. Analyse populations on a smaller geographic scale to further understand the relationship between habitat and population genetic structure. This was undertaken in:
   * The Mediterranean Sea, where these two species coexist, and
   * The eastern coast of South Africa where a different morphotype of the bottlenose dolphin occurs.

The way in which genetic diversity is partitioned within and between populations from different geographical areas is the result of the history and the evolution of a species. To analyse the partition of genetic diversity and therefore to assess the population structure I used two types of molecular markers:

a) the microsatellites, or Simple Sequence Repeat (SSR), and

b) the sequence of the D-loop control region of the mitochondrial DNA (mtDNA),

Microsatellites, are nuclear markers, bi-parentally inherited. Form the molecular perspective they are non-coding sequences of tandem repeat units, widely interspersed in the eukaryotic genome. They are highly informative markers because they have a high level of polymorphism and they are generally considered neutral to any selection pressure.

The mitochondrial DNA is present in a single copy in every eukaryotic cell. In mammals, it is maternally inherited and not subject to recombination. The lack of recombination allows the detection of past evolutionary events such as migrations, bottlenecks, population isolations, by the patterns of coalescence of the different maternal lineages.

The combined analysis of these markers is particularly useful in species with a relatively complex social behaviour. Due to their different mechanism of inheritance, it is possible to track both female and male lineages and therefore assess differences in the pattern of movements of the two sexes and have an insight in the social structure of the species.

The thesis is organised in a general introduction, five main chapters, and a general discussion. Each main chapter is structured as an article. There is little or no change in the format of these articles from the version submitted for publication (as applicable). I believe that any little result or discovery in science should be available as soon as possible to the public in order to have an impact on the scientific progress. I hope that my work will contribute to a better understanding of the species here considered and to the marine life of our oceans.

INTRODUCTION

The bottlenose dolphin is arguably the best known of all cetaceans and a lot of information is available regarding this species. On the contrary, despite its cosmopolitan distribution, information about the common dolphin is relatively scarce.

In comparing the different species of odontocetes two principal characteristics highlight the species under review: their wide and diverse habitat range, and their extremely high morphological variability. The question that arises is what are the evolutionary mechanisms that determined such patterns?

Distribution and Habitat Range

Common and bottlenose dolphins' habitat ranges include all oceans from warm temperate to tropical waters and they show a high adaptability to different environments. The bottlenose dolphin prefers coastal waters and resident populations have been reported throughout its range. However pelagic populations are also known. Conversely, common dolphins are mainly known as pelagic species, although they can also occur in coastal waters.

Morphological variability

The taxonomy of both these species is still uncertain. The exceptional morphological variation in body size, coloration, cranial, and skeletal characteristics in both species have led in the past to the description of several different nominal species, later all were reconsidered local variations of the main species *Tursiops truncatus* and *Delphinus delphis* (Hershkovitz, 1966, Rice 1998). Recently the scientific community agreed the following classification, pending further revisions.

For the bottlenose dolphin two species are currently recognised. *Tursiops truncatus* or the 'common bottlenose dolphin', distributed in most of the world's warm temperate to tropical seas, in coastal as well in offshore waters. *Tursiops aduncus* or 'Indian Ocean bottlenose dolphin', limited to the coastal waters of the Indian Ocean and western Pacific Ocean, from eastern Africa to Taiwan, south-east coast of Australia. However, within *T. truncatus* further differentiation is observed. In the western North Atlantic, coastal and pelagic populations have been found to be

morphologically and genetically differentiated (Mead & Potter, 1995; Hoelzel *et al.*, 1998), and recently it has been suggested that they should be considered different species (Kingston & Rosel, 2003). In the eastern North Pacific, the existence of differentiated nearshore and offshore populations has been reported based on morphological data, (Walker, 1981) and similar differentiation has also been suggested along the South African coasts (Ross, 1977).

For the common dolphin three putative species are recognised. *Delphinus delphis*, or short-beaked common dolphin mostly distributed in the Atlantic and Pacific Ocean, along the coasts and in pelagic waters. *Delphinus capensis*, or long-beaked common dolphin, distributed disjunctly in coastal waters of west Africa, in the western Atlantic from Venezuela to Argentina, in the eastern Pacific from southern California to central Mexico and Peru, in the western Pacific around Korea, southern Japan and Taiwan, and in the Indian Ocean in waters off Madagascar, South Africa, and possibly Oman. The third species, or possible subspecies of *D. capensis*, shows even longer beak morphology, is named *D. tropicalis* and occurs in the north Indian Ocean and south eastern Asia. However, populations with intermediate morphological characteristics have been observed in several regions (Amaha, 1994; Murphy, 2004), questioning whether these morphotypes can be recognised as different species worldwide. Associations of common dolphins with other marine mammal species (*Grampus griseus*, *Stenella sp.* and *Tursiops sp.*) are not uncommon (Jefferson *et al.*, 1993).

In the Black Sea both species are referred to as possible subspecies *Tursiops truncatus ponticus* and *Delphinus delphis ponticus* (Tomilin, 1957; Hershkovitz, 1966). They differ from the main morphotypes because of their smaller body size, different colour pattern, and different life history.

Factors involved in population differentiation

Investigating the reasons of such high morphological variability, several factors can be identified as possible causes of population differentiation. Some are more linked to the characteristics of the environment, others to the intrinsic characteristics of each species.

Geographic distance

Geographic distance has largely been considered a key factor determining intraspecific differentiation especially for terrestrial species. Distant populations can progressively differentiate, because of limited gene flow. Genetic drift and natural selection can act on populations and leading to speciation over time. On land, physical boundaries frequently enhance this process. In the aquatic environment, with no apparent boundaries to the movement of individuals, water temperature, distribution of resources, depth, and current regimes may play similar roles.

Environmental changes determining rarefaction of the habitat may favour isolation and this process has been observed for some cetaceans. The antitropical distribution of some species (eg. *Lagenorincus obliquidens* and *L. obscurus*, Hare *et al.*, 2003; *Lissodelphis borealis* and *L. peronii*, Fordyce, 2002) probably arose allopatrically when populations became isolated either side of the tropics through changing sea temperatures or current regimes.

However, this scenario does not match the pattern observed in bottlenose and common dolphins where all species are widely dispersed across significant distances, habitats and conditions. This widespread distribution may indicate that they coped reasonably well with any climate change that has occurred in the past, probably adapting easily to the new conditions.

Moreover, in several cases different morphotypes are observed in the same geographic area. For example in the area between the Chinese coasts and Taiwan, *T. truncatus* and *T. aduncus* occur sympatrically, but the former prefers pelagic waters, whereas the latter is generally found only in coastal shallow waters (Wang, 1999). Similarly, in the eastern North Pacific *D. delphis* and *D. capensis* live in sympatry, the first preferring more pelagic waters whereas the second confined in coastal shallow waters. Analogous situation is observed in south Japan (Amaha, 1994). Considering that both common and bottlenose dolphins are highly mobile species capable of long distance movements (Lockyer, 1978; Wells *et al.*, 1999; Wood, 1998), it is reasonable to assume that geographic distance is not a factor limiting movements of individuals across regions, at least in the aforementioned cases.

Foraging specialization

Specialization for local food resources is another factor suggested as possible cause of intraspecific population differentiation in cetaceans (Hoelzel, 1998). In the

killer whale specialization on different foraging behaviour was defined as the main factor determining the differentiation between 'resident' populations, principally feeding on fish, and 'transient' populations, mainly feeding on other small marine mammals (Bigg, 1982). Resident and transient killer whales also differ in a variety of morphological, behavioural and genetic aspects (Morton, 1990; Hoelzel *et al.*, 1998). Nearshore and offshore bottlenose dolphins are also known to have different diets. Stomach contents analysis indicated that the nearshore bottlenose dolphins feed mainly on coastal species of fish (sciaenid) or cephalopod (*Loligo sp.*), whereas the offshore dolphins preyed principally on pelagic species of squid and fish (family Myctophidae) (Mead & Potter, 1995).

Information about common dolphin feeding behaviour is scarce. Common dolphins appear to feed opportunistically, their diet reflecting local prey abundance. Prey is made up of primarily pelagic species such as small mesopelagic fish and squid found in the deep scattering layer and epipelagic schooling species such as small scombroids, clupeoids and squid (Evans, 1994; Ohizumi *et al.*, 1998; Silva & Sequira, 1996; Berrow & Rogan, 1995; Boutiba, & Abdelghani, 1995; Relini, & Relini, 1993). No distinction between offshore and nearshore forms have been observed, although in some areas common dolphins can move inshore. Along the eastern coast of South Africa common dolphins occur inshore during winter when they appear to follow the movements of sardines (*Sardinops ocellatus*) up the coast (Peddemors, 1999). In the eastern Mediterranean Sea common dolphins are also recorded in coastal waters and resident coastal populations are also observed (Politi *et al.*, 2001). Systematic comparison of the stomach contents in the different morphotypes of in offshore and nearshore populations has not been conducted.

Different feeding strategies often require a high degree of cooperation and synchrony of action of more than one individual, and therefore they indirectly influence numerous social factors such as group size, group composition and sex ratio. Observations on bottlenose dolphin coastal resident populations indicate extreme diversity in prey and feeding techniques (Hoese, 1971; Rigley, 1983; dos Santos and Lacerta, 1987; Rossback & Herzing, 1997; Smokler *et al.*, 1997). Specialised foraging behaviour is often observed, and succeeding generations of bottlenose dolphins apparently continue to use the same innovative strategies and feed on the same types of food. For examples this is the case of dolphins 'strand feeding' on mullets that are driven onto mud banks or dolphins following working

shrimps boats to acquire discards (Shane, 1990). Fewer details are available for bottlenose dolphins inhabiting offshore waters, although among those populations cooperative feeding is also observed (Würsig & Würsig, 1979; Saayman *et al.*, 1973, Irvine *et al.*, 1981, Wells *et al.*, 1980).

Interactions between common dolphins and human fishing activities have been reported, where common dolphins feed on the fish entangled in the nets or on fish discarded by fishermen (Leatherwood and Reeves, 1983) suggesting that some groups can specialize on particular feeding activities in this species as well.

Social structure

The social structure of a species is generally shaped around its feeding behaviour, life history, mating strategies and habitat. The type of social structure adopted by a species aims to maximize the individual success through the success of the group. In the marine environment, it has been generally observed that pelagic populations have a bigger group size than coastal populations and this has also been observed in odontocetes (Wells *et al.*, 1980). Bigger groups can be advantageous in finding food resources in a dispersed environment like the open oceans. Larger groups also offer better protection against predators.

The social structure of coastal bottlenose dolphins has been widely studied in different parts of the world. Bottlenose dolphins show a complex social structure with different levels of aggregations (pods, groups, herds, super alliances; Connor, 2000). They are generally observed in groups of 2-15 individuals. Group composition tends to be dynamic, with sex, age, reproductive conditions, familial relationships and affiliation histories apparently being the most important determining factors (Wells, 1991). Subgroups may be stable or repeated over periods of years. Basic social units include nursery groups, mixed sex groups of juveniles, and adult males as individuals or strongly bonded in pairs or trios (Connor *et al.*, 2001).

Not much is known about common dolphin social structure. Groups of hundreds or thousands are generally observed in offshore waters. Evans (1994) suggested these schools to be composed of smaller subgroups of 20-30 individuals. Moreover, he suggested that segregation may be based on age and sex, but no systematic observations have proven so. Difference between groups in colour pattern

and cranial measurements suggest that groups may have temporal integrity at some level.

Life history

A life history is the set of features in an organism's life cycle, especially those affecting survivorship and reproduction. Body size is a good predictor of life history and generally bigger organisms have longer generation time and lower reproductive rates. Both bottlenose and common dolphins, as all cetaceans, are K-strategists characterised by long generation time, low reproductive rate, and long nursing periods. For bottlenose dolphins it has been demonstrated that females can live more than 50 years (Hohn *et al.*, 1989) and some males have reached 40-45 years (Wells & Scott, 1999). For common dolphins lifespan has been estimated to be around 20 years. Sexual dimorphism has been observed in some populations of common dolphins, with males generally slightly bigger than females. It has been suggested that this could be linked to different mating strategies in different populations (Amaha, 1994). Age at sexual maturity varies by regions and generally females achieve it before males. Sexual maturation for females and males in bottlenose dolphins is achieved at 5-13 years and 9-14 years, respectively, whereas for common dolphins is achieved at 6-7 years and 7-12 years. Calving interval is between 1-3 years, although in free-ranging bottlenose dolphins this period can be prolonged up to 5-6 years. Generally, separation from the mother coincides with the birth of the next calf.

In *T. aduncus* sexual maturity may be reached at an older age with female producing their first calf at the age of 12 or older and calving interval is generally longer (Connor *et al.*, 2001). For the Black Sea common dolphins, sexual maturation is reached at a younger age and the calving interval is shorter (Tomilin, 1957).

The hypotheses

The marine environment has had and still has an enormous influence on the evolution of cetaceans. On the broadest scale there are two main habitats, coastal and pelagic. Coastal habitats are characterised by high variability in environmental conditions, even across small geographic ranges, and physical boundaries can be frequent. Pelagic habitats are more homogeneous with no apparent boundaries (though relevant boundaries may in fact exist). The hypothesis is whether the habitat

structure may drive the population structure of a species and therefore its evolutionary history.

Bottlenose and common dolphins are closely related species, evidenced by their still controversial taxonomic status (Le Duc *et al.*, 1999). They both belong to the subfamily *Delphininae* that rapidly radiated generating numerous different species. On average, they show similar geographic distribution. However, common dolphins are mostly pelagic, although coastal populations are observed in some areas, whereas bottlenose dolphins are mostly coastal, with some pelagic populations. If the habitat has an impact in shaping the population structure of a species, I expect to see greater population structure in a coastal species, such as the bottlenose dolphin, that in a pelagic species, such as the common dolphin. Exceptions may be seen in each species in the less common habitat for that species.

Each of these species show a high degree of morphological differentiation across their range, in some cases leading to the classification of proposed new species. If local habitat differences are driving genetic and phenotypic differentiation, then there may not be a direct correlation between morphotype and genetic structure, especially for similar morphotypes in geographic isolation but similar habitat. In this case similar phenotypes may have diverged in allopatry while different phenotypes may diverge in parapatry.

THE BOTTLENOSE DOLPHIN

Tursiops truncatus

Tursiops aduncus

CHAPTER 2

Population structure and speciation in the genus *Tursiops* based on microsatellite and mitochondrial DNA analyses

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Running title: bottlenose dolphin population genetics

Abstract

Bottlenose dolphins (*Tursiops truncatus*) have a world-wide distribution, and show morphotypic variation among regions. Distinctions between coastal and pelagic populations have been documented, however regional patterns of differentiation had not been previously investigated in a wider geographic context. We analysed up to 9 different populations from 7 different areas of the world by mitochondrial DNA and microsatellite DNA markers, and found differentiation among all putative regional populations. Both mtDNA and microsatellite DNA data show significant differentiation, suggesting restricted gene flow for both males and females. Dolphins in coastal habitat showed less variability and were in most cases differentiated from a pelagic lineage, which could suggest local founder events in some cases. Two coastal populations recently classified as belonging to a new species, *T. aduncus*, were each highly differentiated from populations of the *truncatus* morphotype, and from each other, suggesting a possible third species represented by the South African aduncus type.

Keywords: bottlenose dolphins, population genetics, speciation, microsatellites, mtDNA, molecular ecology, phylogeography

Introduction

The evolutionary radiation of species is directly related to the pattern of diversity within species, and the forces that generate those patterns. In the marine environment there are relatively few boundaries of the type that can lead to differentiation by drift in territorial species (such as rivers and mountains). For some species, especially highly mobile marine vertebrates such as teleost fishes, the pattern of genetic variation can be effective panmixia across large geographic regions (see review by Graves, 1996). However, marine mammals often show fine-scale population structure, though the extent varies among species (see review in Hoelzel *et al.*, 2002). Hoelzel (1998a) has argued that this could be due to a combination of behavioral specializations for local resources, social structure and in some cases historical environmental change. In this study we investigate the pattern and forces leading to population structure in a highly mobile, social marine species, the bottlenose dolphin.

Tursiops is a polytypic genus, which in the past has been divided into as many as 20 different species (Hershkovitz, 1966), though often based on very limited data. The more persistent classifications included *T. gilli* and *T. nuuanu* in the eastern North Pacific (Walker, 1981) and *T. aduncus* (Ross, 1977; Ross & Cockcroft, 1990) in Australia, the Indian Ocean, China and South Africa. Morphotypes differ in colour pattern, body dimension and cranial structure, though character distributions typically overlap (Walker, 1981; Ross & Cockcroft, 1990). As a consequence, only the single species *T. truncatus* was recognised (Ross & Cockcroft, 1990; Wilson & Reeder, 1993) until molecular data supported the separate classification of *T. aduncus* (LeDuc *et al.*, 1999; Wang *et al.*, 1999). This pattern is not uncommon among delphinid cetaceans (e.g. similar morphotypic diversity is seen in *Orcinus orca*: Evans *et al.*, 1982, Visser & Makelainen, 2000; *Stenella longirostris*; Perrin *et al.*, 1991 and *Delphinus delphis*; Jefferson & Van Waerebeek, 2002). However, it remains unclear to what extend these are polytypic species or clusters of closely related species (but see Hoelzel *et al.*, 2002).

The bottlenose dolphin has a wide distribution in both hemispheres, from cold temperate to tropical waters. In some parts of its range there is a clear distinction between populations in coastal and pelagic habitat, though this has not been fully explored in many locations. Parapatric coastal and pelagic populations sometimes differ in morphology, prey choice and parasite load (Mead & Potter, 1995; Hoelzel *et*

al., 1998a), but the distinction varies among geographic regions (Walker, 1981; Mead & Potter, 1995). In most parts of its range, *T. aduncus* is found in coastal habitat, and is distinguished from *T. truncatus* by a smaller overall size, spotted ventral and lateral pigmentation, and an elongated beak, among other characters (Ross, 1977). Coastal and pelagic populations described in Chinese waters around the Penghu Archipelago were identified as *T. aduncus* (coastal form) and *T. truncatus* (pelagic form) (Gao *et al.*, 1995). Wang *et al.* (1999) compared these populations using 5' mitochondrial DNA (mtDNA) control region sequence and found a nucleotide divergence of 4.4%, 6 fixed nucleotide differences, and reciprocal monophyly. These data, together with the inclusion of *T. aduncus* in a delphinid phylogeny based on the entire mtDNA cytochrome b gene (LeDuc *et al.*, 1999), support the reclassification of *aduncus* morphotypes at the species level at least.

Coastal and pelagic populations in the western North Atlantic (from Florida north to Nova Scotia) have been compared for morphology, feeding ecology, parasite load (Mead & Potter 1995), hemoglobin profile (Hersh & Duffield, 1990), microsatellite DNA, and mtDNA control region diversity (Hoelzel *et al.*, 1998a). In each case distinctions were evident. The genetic differentiation between these populations was less than that seen between *T. truncatus* and *T. aduncus* in China (Wang *et al.*, 1999). Putative populations on either side of Florida were also compared and found to be differentiated (based on mtDNA RFLP analysis), though it is not clear if all samples compared were of the same morphotype (i.e. all coastal or all pelagic; Dowling & Brown, 1993).

In this study we test the hypothesis that the local fine-scale population structure found in the western North Atlantic for *Tursiops truncatus* (Hoelzel *et al.*, 1998a) is characteristic of populations in this genus throughout its range. Towards this end we greatly extend the representation of regional populations in the Atlantic Ocean, and include a comparison of *aduncus*-type dolphins from South Africa with the published *T. aduncus* sequences from China, and with data for the common dolphin (*Delphinus delphis*). A sample of *T. truncatus* from the eastern North Pacific is also included. Our further objective is to address the question of how population structure may have evolved in a highly mobile marine vertebrate species, given the pattern of differentiation observed. We find differentiation among all regional populations, with the strongest differences between the South African *aduncus*-type samples and all others (including the published Chinese *T. aduncus*

sequences). The pattern shows a distinction between two highly polymorphic pelagic populations (one in the North Atlantic and one in the North Pacific) and regional coastal populations that differ from pelagic populations to varying extents, and often show less polymorphism. The implication is that structure has evolved as a result of philopatry and historical founder events, and that behavioral strategy and historical environmental factors are likely both important.

Materials & methods

Sample collection and DNA extraction

In total, 269 *Tursiops* sp. samples from 7 geographic regions were analysed in this study (see Table 1 and Fig. 1).

Table 1. List of the populations analysed and correspondent acronyms. The number of samples for each population considered in this article are reported for the microsatellite and the mtDNA analyses. Data taken from other publications are as follows: a) for these two populations data for five of the microsatellite loci are from Hoelzel *et al.* (1998a) (see text for details). b) from Hoelzel *et al.* (1998a). c) one sequence is from Wang *et al.* (1999), the rest are from Hoelzel *et al.* (1998a). d) 5 of these sequences are from Hoelzel *et al.* (1998a). e) from Wang *et al.* (1999).

Population	Acronym	Microsatellite DNA	mtDNA
Mediterranean Sea	**MS**	45	18
Eastern North Atlantic	**ENA**	27	9
Western North Atlantic pelagic	**WNAP**	27[a]	25[b]
Western North Atlantic coastal	**WNAC**	27[a]	29[b]
Eastern North Pacific	**ENP**	14	1[b]
Gulf of Mexico	**GM**	22	10
West Africa	**WA**	-	16[c]
Bahamas	**BAH**	-	4[b]
South Africa	**SA**	107	38[d]
Chinese *truncatus*-type	**CHt**	-	17[e]
Chinese *aduncus*-type	**CHa**	-	19[e]
Delphinus delphis		30	15

Samples from South Africa (SA) are from a coastal population described as *T. aduncus*, while all other samples are from individuals described as *T. truncatus*. Most of the samples were obtained from stranded dolphins or dolphins caught in nets. Some samples from the Mediterranean Sea (MS) and SA were from biopsy sampling as part of long-term population studies. Samples from MS were from 7 different regions covering different areas of the basin (all sampled in coastal habitat).
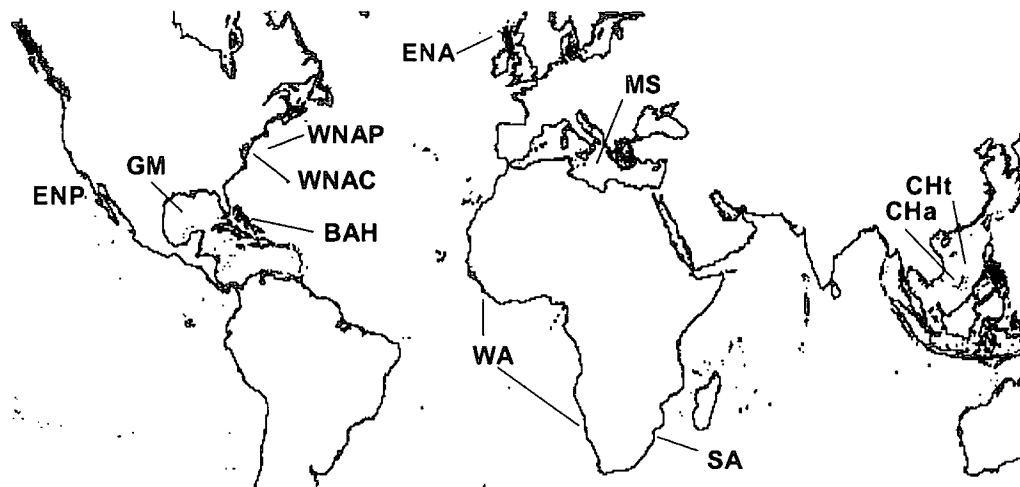
Figure 1: Map of sample locations. Abbreviations are as in Table 1.

Eastern North Atlantic (ENA) samples were from strandings (presumably coastal animals) from the east and west of Scotland and the south of England. Samples from coastal (WNAC) and pelagic (WNAP) populations in the western North Atlantic are from Hoelzel *et al.* (1998a) and were verified as belonging to the respective coastal and pelagic populations as described in Hoelzel *et al.* (1998a). Eastern North Pacific (ENP) samples were from California (from strandings and probably from coastal habitat, but this is not known). Samples from the Gulf of Mexico (GM) are all from stranded animals collected between Galveston and Corpus Christi, Texas. While direct confirmation for these samples was not possible, they are likely to represent the coastal stock since morphometric studies have classified 98.5% of 205 stranded samples from this region as 'coastal' morphotype (Turner, 1998).

DNA was extracted from tissue samples preserved in salt saturated 20%DMSO by a standard phenol/chloroform extraction method (Hoelzel, 1998b).

Previously published mtDNA sequences were included for comparison of some populations including pelagic *T. truncatus* from waters around Taiwan and Hong Kong (CHt), coastal *T. aduncus* from Taiwan, Indonesia, and Beihai (CHa) in southern China (Wang *et al.*, 1999), and western Africa from Namibia to Mauritania (WA; Hoelzel *et al.*, 1998a, Wang *et al.*, 1999). Most of the latter samples were from strandings and the source populations unknown, though a few were known to be from pelagic populations. Sequences from coastal animals from the Bahamas (BAH) were also used (Hoelzel *et al.*, 1998).

Microsatellite analysis

Nine published microsatellite loci were analysed for all 269 samples, with the exception of the WNAC and WNAP populations where data for 5 of the 9 microsatellites (KWM1b, KWM2a, KWM2b, KWM9b, KWM12a) were taken from Hoelzel *et al.* (1998a). All other samples are analysed here for the first time (Table.1).

Primers KWM1b, KWM2a, KWM2b, KWM9b, KWM12a were derived from *Orcinus orca* (Hoelzel *et al.*, 1998b), EV37Mn from *Megaptera novaeangliae* (Valsecchi & Amos 1996), TexVet5, TexVet7 and D08 from *Tursiops truncatus* (Rooney *et al.*, 1999; Shinohara *et al.*, 1997). Amplified DNA was analysed for length variation on 6% polyacrylamide denaturing gels using fluorescent imagining on an automated ABI PRISM 377 DNA sequencer (Applied Biosystem, Warrington,UK), after incorporation of 1/10 fluorescent labelled primer (PCR reaction conditions: 100μM dNTPs, 0.75-1.5 mM MgCl$_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 200nM of each primer, 0.02 U/μL Taq polymerase. PCR cycling profile: 5 min at 95°C; then 35 cycles of 40 sec at 94°C, 1min at the T°ann, 1 min at 72°C; then 10 min at 72°C). The annealing temperatures (T°ann) were as follows: KWM1b: 45°C; KWM2a: 43°C; KWM2b: 44°C; KWM9b: 55°C; KWM12a: 46°C; EV37Mn: 57°C; TexVet5: 54°C; TexVet7 and D08: 57°C. An internal standard marker (Genescan-500 ROX, Applied Biosystems, Warrington,UK) was used to determine the allele sizes.

A closely related species, *Delphinus delphis*, was analysed to better clarify the relationship between the two *aduncus*-type and the *truncatus*-type populations. The same nine microsatellite loci were used to screen 30 *D. delphis* samples from different geographical areas (MS, ENA and ENP). This sample set was compared against all *T. truncatus* populations grouped together (162 samples), and the SA population (107samples).

For microsatellite loci, the level of polymorphism was estimated as the number of alleles per locus, observed heterozygosity (H$_o$), expected heterozygosity (H$_e$), and allelic richness. Allelic richness controls for variation in sample size by a rarefaction method, and was calculated using the program FSTAT 2.9.3 (Goudet, 2001). Evaluation of possible deviations from the expected Hardy Weinberg genotypic frequencies (overall deviation, heterozygote deficiency and heterozygote excess) and linkage disequilibrium were performed using Fisher's exact test and the Markov

chain method (dememorization number, number of batches, iteration per batch set at 1,000, Bonferroni correction applied). These analyses were performed using GENEPOP 3.1d (Raymond & Rousset, 1995a,b). Genetic differentiation among populations was assessed based on both the infinite allele model (IAM) using F_{ST}, and the stepwise mutation model (SMM) using Rho_{ST}. The level of differentiation between population pairs was estimated as F_{ST} (Weir & Cockerham, 1984) using the program FSTAT 2.9.3 and Rho_{ST} using the program RstCalc (Goodman, 1997). The significance of the difference of F_{ST} and Rho_{ST} values from zero was tested by permutation analysis, and the Sequential Bonferroni correction (Holm, 1979) was applied using the program Multiplicity (Brown & Russel, 1996). A permutation test to assess differentiation for allele size was performed comparing F_{ST} and Rho_{ST} using the program SPAGeDi (Hardy and Vekemans, 2002). Genetic distances between populations were estimated using Nei's Da genetic distance (Nei, 1987). Calculations were performed using GenDist (http://www.biology.ualberta.ca/jbrzusto/GeneDist.html). The distance matrix was used to reconstruct un-rooted Neighbor Joining trees as implemented in PHYLIP version 3.56 (Felsenstein, 1993).

mtDNA analysis

The first 297bps at the 5' end of the mtDNA control region were sequenced in a total of 70 samples, while further sequences were obtained from the published databases. In total 186 sequences of *Tursiops* sp. were available (see Table.1).

The mitochondrial DNA control region was amplified with universal primers MTCRf (5'-TTC CCC GGT GTA AAC C) and MTCRr (5'-ATT TTC AGT GTC TTG CTT T) after Hoelzel (1998b). The PCR reaction conditions were as follows: 100μM dNTPs, 1.5 mM MgCl$_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 200nM of each primer, 0.02 U/μL Taq polymerase. The PCR cycling profile was 4 min at 95°C, 35 cycles of 45 sec at 94°C, 1.5 min at 50°C, and 1.5 min at 72°C, followed by 8 min at 72°C. PCR products were purified with QIAgen PCR purification columns (QIAGEN, GmbH, Germany) and sequenced directly using the ABI dye-terminator method. Five samples were sequenced in both directions, and no ambiguities were found. A total of 15 *D. delphis* haplotypes including 2 from MS, 5 from ENA (sequenced for this study) and 8 from ENP (from Rosel *et al.*, 1994) were also

included. Sequence alignment was performed using SEQUENCHER 3.0 (Gene Code Corp.).

The degree of differentiation (F_{ST} and Φ_{ST}) and Tajima's D were estimated using ARLEQUIN 2.0 (Schneider *et al.*, 1999). Estimates of Φ_{ST} used the Tamura-Nei genetic distance model (Tamura & Nei, 1993) with a gamma correction of $\alpha = 0.47$ (as estimated for the 5' hypervariable segment of the human control region by Wakeley, 1993). Genetic distance (Da) was estimated using Tamura-Nei with the SENDBS programme, written by N. Takezaki (National Institute of Genetics, Mishima, Shizuoka, Japan; http://oat.bio.indiana.edu:7580/documents/public/molbio/tools/Sendbs/). SENDBS was also used to estimate the nucleotide diversity (π) in each population. Populations were compared using Da by neighbour joining in PHYLIP (un-rooted trees), as for the microsatellite DNA data, and the two consensus trees compared for congruence using the quartet method and the program QUARTET (Estabrook, 1992).

Individual haplotypes were compared phylogenetically by the neighbour-joining method using PAUP* 4.0b10 (Swofford, 1997) and rooted with homologous sequence from the killer whale (*Orcinus orca*). Majority-rule consensus trees were constructed from 1,000 bootstrap replications and a 50% criterion for the retention of nodes was applied. Distances were based on Tamura-Nei as above. The ti/tv ratio was set at 6:1, based on observed values. A maximum parsimony phylogenetic reconstruction was based on 1,000 bootstrap replications, retaining branches with 50% support or greater.

A median-joining network was generated to infer phylogenetic relationships among the Atlantic and Mediterranean mtDNA haplotypes (ENA, MS, WA, WNAC, WNAP and GM), using the program NETWORK 2.0 (Bandelt *et al.*, 1999; www.fluxus-engineering.com).

Results

Microsatellite results

Each pair of loci was tested for linkage disequilibrium and genotypic independence was confirmed. Expected (H_e) and observed (H_o) heterozygosity values for each locus are reported in Table.2. Hardy-Weinberg equilibrium was tested for each population at each locus. Only the Mediterranean population deviated

significantly from the HW genotypic proportions (χ^2 = 96.5, d.f. = 18, p=0.000) and a significant heterozygote deficiency was found for two loci (Table 2). Omission of these loci did not significantly change the pattern of differentiation between MS and other populations, so they were retained for the results presented below. No significant heterozygote excess was observed at any locus in any population.

Table 2. Number of alleles (number of private alleles in parentheses, allelic richness in square brackets), expected (He) and observed (Ho) heterozygosities for each population at each microsatellite locus. The asterisks indicate those loci with a p-value < 0.00079 (Bonferroni correction applied) when tested for heterozygote deficiency. Abbreviations are as in Table 1.

Loci		Populations						
		truncatus						aduncus
		MS N=45	ENA N=27	WNAP N=27	ENP N=14	WNAC N=27	GM N=22	SA N=107
KWM1b	N° of Alleles	4 (2) [2.8]	2 [1.9]	3 [1.8]	3 (1) [2.8]	2 [2.0]	2 [2.0]	4 (2) [2.4]
	Ho	0.067*	0.080	0.083	0.250	0.348	0.227	0.505
	He	0.248	0.187	0.122	0.308	0.329	0.312	0.524
KWM2a	N° of Alleles	10 (2) [6.1]	4 [3.7]	7 [6.5]	5 [4.7]	4 [3.8]	5 [4.3]	4 (1) [3.0]
	Ho	0.704	0.704	0.708	0.615	0.680	0.762	0.364
	He	0.761	0.658	0.837	0.732	0.607	0.669	0.465
KWM2b	N° of Alleles	7 (1) [3.3]	3 [2.9]	5 [3.5]	3 [2.9]	2 [1.7]	3 [2.6]	2 [1.9]
	Ho	0.267	0.4	0.348	0.461	0.087	0.176	0.215
	He	0.318	0.473	0.346	0.480	0.127	0.315	0.201
KWM9b	N° of Alleles	6 [4.3]	5 [3.1]	6 [5.7]	4 [3.7]	5 [4.0]	3 [2.7]	6 (3) [4.3]
	Ho	0.439	0.538	0.8	0.583	0.538	0.333	0.757
	He	0.597	0.581	0.818	0.616	0.667	0.426	0.708
KWM12a	N° of Alleles	12 (1) [7.3]	9 [6.2]	10 (1) [7.5]	4 [3.9]	5 [4.4]	4 [3.9]	11 [6.3]
	Ho	0.710	0.696	0.667	0.545	0.667	0.368	0.755
	He	0.798	0.75	0.807	0.515	0.72	0.654	0.770
EV37Mn	N° of Alleles	24 (3) [12.8]	12 [8.6]	17 (1) [11.9]	4 [6.4]	13 [8.2]	14 [10.6]	15 [6.3]
	Ho	0.923	0.615	0.917	0.692	0.778	0.954	0.743
	He	0.947	0.848	0.926	0.769	0.815	0.921	0.811
TexVet5	N° of Alleles	5 [4.4]	5 [4.0]	9 [7.6]	6 (1) [6.3]	6 [4.6]	4 [4.0]	6 (1) [4.8]
	Ho	0.567	0.611	0.833	0.643	0.63	0.4	0.711
	He	0.681	0.571	0.871	0.754	0.699	0.6	0.696
TexVet7	N° of Alleles	9 (1) [4.8]	4 [3.2]	8 (1) [6.4]	5 (1) [4.4]	6 [4.1]	5 [4.3]	4 [2.3]
	Ho	0.364*	0.593	0.731	0.571	0.63	0.762	0.449
	He	0.570	0.543	0.812	0.593	0.66	0.711	0.506
D08	N° of Alleles	11 (2) [6.4]	4 [3.7]	11 (1) [7.7]	4 [3.7]	7 [4.4]	4 [3.9]	4 [1.7]
	Ho	0.698	0.461	0.808	0.461	0.667	0.667	0.075
	He	0.785	0.495	0.869	0.548	0.6	0.679	0.082
Average (± s.d.)	N° of Alleles	9.8 ± 6.0 [5.8 ± 3.0]	5.3 ± 3.2 [4.2 ± 2.0]	8.4 ± 4.1 [6.5 ± 2.8]	4.2 ± 1.0 [4.3 ± 1.3]	5.6 ± 3.3 [4.1 ± 1.8]	4.9 ± 3.6 [4.3 ± 2.5]	6.2 ± 4.1 [3.7 ± 1.8]
	Ho	0.527 ± 0.266	0.522 ± 0.193	0.655 ± 0.268	0.536 ± 0.132	0.558 ± 0.213	0.517 ± 0.275	0.508 ± 0.254
	He	0.633 ± 0.228	0.567 ± 0.187	0.712 ± 0.279	0.591 ± 0.150	0.580 ± 0.216	0.587 ± 0.201	0.529 ± 0.252

Comparisons among populations showed higher average allelic diversity and heterozygosity in WNAP and MS than any of the other populations (Table 2). In the North Atlantic, allelic richness was significantly greater in the pelagic WNAP sample than in the coastal WNAC, GM & ENA samples combined (Mann-Whitney U-test, Z=-2.14, p=0.032), but WNAP was not significantly different from MS (nor were WNAC, GM or ENA significantly different from each other). A similar pattern is seen for heterozygosity where average H_o is significantly higher for WNAP than WNAC, GM & ENA combined (Mann-Whitney U-test, Z=-1.96, p=0.05), and WNAP was not significantly different from MS.

Genetic differentiation among pairwise populations was estimated using F_{ST} and Rho_{ST}. The results obtained with the two methods both show significant differentiation for all pairwise comparisons (Table 3), including the comparison between coastal samples from either side of Florida (WNAC v GM).

Table 3. Genetic differentiation among pairwise populations using microsatellite data. F_{ST} values are reported below the diagonal while Rho_{ST} values are reported above the diagonal. All the F_{ST} and Rho_{ST} values are significantly different from zero (p<0.05, or * for p<0.0001).

	N	MS	ENA	WNAP	ENP	WNAC	GM	SA
MS	45		0.048	0.034	0.353*	0.196*	0.161*	0.345*
ENA	27	0.098		0.161*	0.460*	0.367*	0.314*	0.540*
WNAP	27	0.064	0.116		0.272*	0.236*	0.251*	0.392*
ENP	14	0.283	0.288	0.219		0.511*	0.555*	0.710*
WNAC	27	0.221	0.282	0.205	0.270		0.060	0.576*
GM	22	0.224	0.282	0.199	0.281	0.060		0.526*
SA	107	0.293	0.273	0.260	0.364	0.345	0.317	

The smallest values, though still significant, were seen between WNAP and MS. The SA population showed the highest differentiation compared to all the other populations. The data suggest relative similarity between two clusters of putative populations, MS, ENA and WNAP for one, and WNAC and GM for the other. A comparison between F_{ST} and Rho_{ST} to assess the role of allele size in population differentiation (after Hardy *et al.*, 2003) indicated no significant role for allele size. The phylogeny comparing populations based on Da distances and a neighbour joining analysis was also consistent with the grouping indicated by the F_{ST} and Rho_{ST} analyses (Fig. 2).
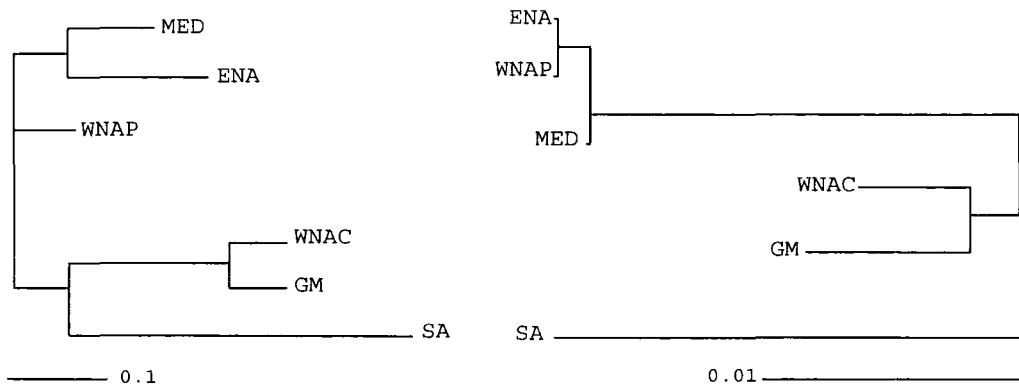
Figure 2: Neighbor Joining trees based on Da distances for microsatellite data (right) and mtDNA data (left). The names of the populations correspond to those given in Table 1.

MtDNA sequence analysis

Mitochondrial DNA control region sequences were compared among the 70 samples sequenced for this study (see Table 1) and in comparison with database sequences representing western Africa (WA) (Wang *et al.*, 1999; Hoelzel *et al.*, 1998a), the Bahamas (BAH) (Hoelzel *et al.*, 1998a) and China, where two populations had been described, one as *aduncus*-type (CHa) and the other as *truncatus*-type (CHt) (Wang *et al.*, 1999).

Sixty six haplotypes were identified showing 56 polymorphic sites (Fig. 3). Shared haplotypes between putative populations were uncommon, observed for three haplotypes among the WNAP, MS, ENA and WA populations, and for one haplotype among GM and BAH. The alignment showed fixed differences distinguishing the SA *aduncus*-type, Chinese *aduncus*-type and *truncatus*-type haplotypes (Fig. 3).

Average gene and nucleotide diversities were estimated for each population. Diversities were relatively high for MS (gene and nucleotide diversities, respectively: 0.94; 0.023), WNAP (0.88; 0.022), Chinese pelagic *truncatus*-type (0.92; 0.024) and WA (0.73; 0.023) populations, and relatively low for the coastal ENA (0.42; 0.016), WNAC (0.43; 0.018), SA (0.29; 0.008), GM (0.72, 0.013) and CHa (0.88, 0.015). Tajima's D was large and negative for three of the populations (ENA: -0.97; WNAC: -1.22; SA: -1.57), suggesting possible population expansion, although it was only significant at the 0.05 level (Beta distribution approximation) for the SA population.

```
                    Polymorphic sites                                    Haplotype frequencies
                1111 1111111222 2222222222 2222222222 222222      MS  ENA WNAP ENP  WA   CHt WNAC GM  BAH SA  CHa
            1224477 8899990133 3556889012 2333445555 5666666677 778899   18   9   25   1   16   17   29   10   4   38   19
            1383143539 6812670402 3146786173 8146782456 7012356713 676837
ENA1        GTCTCCTGTT AATGGCATTC ATGTCCACTA CGCTTACTAT CTCCTCCTTA TCGTTT      1--------------2
ENA2        .......... .......... .......... T......... .C.T...... .T....       1
MS1         .........C .......... .....T.... .......... ..T....... ......       3---7---1--------2
MS2         .A.......C .......... .....T.... .......... ..T....... ......    1
MS3         .......... .......... .......... .........C .......... ..A...    1
MS4         .......... .......... .......... T......... ...T...... .T....       1--------------8
MS5         .......... .......... .......... T......... ...T...... ......    1
MS6         .......... .......... ....T..... T......... ...T...... .T....    1
MS7         ...C...... ..C......T .......... T....C.... ..T....... ..A...    2
MS8         .A........ .......T..T .....T.... T...CCA... ..T....... ..A...    1
MS9         .......... .......T.. .....T.... T...CCA... ..T....... ..A...    1
MS10        .......... .......T.. .....T.... T...CCA... ..T..T.... ......    3
MS11        .......... .......... .......... T......... ...T...... .T....    1
WNAPa       ...C...... ..C......T .....T.... T....CT... .....T.... ..A...             8
WNAPd       .....T.... .......... .......... .......... .....T.... ......             2
WNAPx       .......... .......... .......... T......... ..-..T.... ......             3
WNAPw       .......... G......... .......... C.T....... -..T...... ......             3
WNAPy       .....T.... .......... .......... .G........ .....T.... ......             1
WNAPv       .......... .......... .....T.... T......... .......... ......             2
WNAPe       .......... .......... .......... .......... ...T...... ......             2
WNAPθ       .......... .......... .......... T......... -..T...... .T....             1
WNAPj       ...C...... ..C......T .......... .....CT... ..T....... ..A...             1
WNAPz       ...C...... ..C......T .......... T.T..C.... .......... ..A...             1
WAs         .......... .G.....C.T .......... T....T.... .......... ..A...                        3
WAn         ...C...... ..C......T ....T..... .....C.... .......... ..A...                        1
ENPm        .......... .......... .......... .......T... ...TT..... C.A...                   1
WNACh       .......A.. ...A.T.... .....T.... .....C.... ..TTCT...G ......                            2
WNACc       .......A.. ...A.T.... .....T.... T....C.... ..TTCT...G ......                           22
WNACu       .......A.. ...A.T.... .....T.... ...G.C.... ..TTCT...G ......                            1
WNACg       .......A.. ...A.T.... .....T.... T....C.... ..TTCT....                                  1
WNACi       .......... ...A..G..T .....T.... .....C.... T.TTC.T-.. ..A...                            2
WNAPb       ..T....... ...A...... ...G.T.... .....C.... T.TTCT.... ..A...                            1
BAHk        ..T....... ...A...... .....T.... .....C.... T.TTCT.... ..A...                                      1--------1
BAH1        ..T...G... ...A...... .....T.... .....C.G.. T.TTCT.... ..A...                                               1
GM2         .......... ...A..G..T .....T.... ....CC..G. T.TTC.T... ..A...                                      1
GM3         .......... ...A..G..T .....T.... .....C.... T.TTC.T... ..A...                                      4
GM4         .......... ...A..G..T .....T.... .....CT... T.TTC.T... ..A...                                      2
GM5         .......... ...A..G..T .....T.... .....C..G. T.TTC.T... ..A...                                      1
GM6         .......... ...A..G..T .....T.... .....C.... T.T.C.T... ..A...                                      1
CHt2        ...C...... ..C......T .....T.... T....C.... .......... ..A...                       5
CHt3        .......... ...A....T .......... T...CC.... .C.T.T.... ..A...                        1
CHt5        ...C...... ..C......T G......... TA...C.... .......... ..A.C.                        1
CHt6        .......... .......T.. .....T.... .....T.... ..TTCTT... ..A...                        1
CHt7        ...C.T.... ..C......T .....T.... .....C.... .....T.... ..A...                        1
CHt8        ...CGTC... ..C......T .......... .....C.... .....T.... ..A...                        1
CHt9        ...C...... ..C......T G......... T....C.... .......... ..A.CC                        1
CHt10       ...C.T.... ..C......T .......... .....C.... .....T.... ..A...                        1
CHt11       ...CG..... ..C......T .......... TA...C.... .....T.... ..A...                        2
CHt12       ....G..... ....A....T .......... T...CC.... .C.T.T.... ..A...                        1
CHt13       ...C...... ..C......T .....T.... T....C.... ..T....... ..A...                        1
CHt14       ...CG..... ..C......T .....T.... T....C.... .......... ..A...                        1
SA1         A.......C ...A....T .....T.... .A........ T.TTCT...G ..A...                                               3
SA2         A.......C ...A....T .....T.... .A...G.... ..TTC....G ......                                               1
SA3         A.......C ...A....T .....T.... .A........ ..TTCT...G ......                                              32
SA4         A.......C ...A....T .....T.... .A........ T.TTCT..CG ..A...                                               1
SAt         A.......C ...A....T .....T.... .A........ ..TTCT...G C.....                                               1
CHa1        A...T..CC ...A....T ....T.TT.. ........G. T.TTC..C.. ......                                                      2
CHa2        A...T..CC ...AA...T .C...TT.. ........G. T.TTCT.C.. ..AC..                                                      6
CHa3        A...T..CC ...AA...CT ......TT.. ........G. T.TTC..... ..AC..                                                      1
CHa4        A...T..CC ...A....T ....T.TT.. ........G. T.TTC..C.. ..A...                                                      1
CHa5        A...T..CC ..CAA....T .CA.T.TT.. ........G. T.TTC..C.. ..AC..                                                      1
CHa6        A...T..CC ...AA...T .C..T.TT. .......GT. T.TTC..... ..AC..                                                      1
CHa8        A...T..CC ...AA...T .C...T... T......G.. T.TTCT.C.. ..A...                                                      3
CHa9        A...T..CC ...A....T ....T.TT.. T......G.. T.TTC..C.. ......                                                      2
CHa10       A...T..CC ...AA...CT ......TT.. ........G. T.TTC..C.. ..AC..                                                      1
CHa11       A...T..CC ...AA....T .C...TT.. ........G. T.TTCT.... ..AC..                                                      1
```

Figure 3: Polymorphic sites among 66 haplotypes are shown (left). Position 1 corresponds to the position 15,906 of the *Balaenoptera musculus* mtDNA sequence (Arnason *et al.*, 1993). Haplotypes were identified by an abbreviation for their geographic region (in capital letters – see text). Further small letters and numbers identify the name of the same sequence as published in previous publications. Dots indicate identity with the reference sequence. Straight-line vertical boxes indicated fixed mutations or deletions between sequences from truncatus-type and aduncus-type animals. Dashed-line vertical boxes indicated fixed mutations within the aduncus-type. Haplotype frequencies (right) were reported for each haplotype in each putative population. Horizontal dashed-line boxes indicated shared haplotypes among populations.

Genetic differentiation among pairwise populations was estimated using F_{ST} and Φ_{ST} (Table 4). All pairwise comparisons showed significant differentiation, consistent with the pattern obtained with the microsatellite data. We also found a

significant correlation between the F_{ST} and Φ_{ST} matrices applying the Mantel test (d.f.=8, p=0.02). Significant correlation was also found between the mtDNA and microsatellite DNA F_{ST} matrices (Mantel test, d.f.= 4, p=0.002).

Table 4. Genetic differentiation among pairwise populations using mtDNA data. F_{ST} values are reported below the diagonal while Φ_{ST} values are reported above the diagonal. All the values are significantly different from zero (p<0.05, or * = p<0.001, **= p<0.0001).

	N	MS	ENA	WNAP	WA	CHt	WNAC	GM	SA	CHa
MS	18		0.171	0.089	0.089	0.320**	0.644**	0.555**	0.784**	0.726**
ENA	9	0.189*		0.322	0.341	0.574**	0.796**	0.724**	0.904**	0.805**
WNAP	25	0.089**	0.286**		0.138	0.215**	0.647**	0.569**	0.778**	0.731**
WA	16	0.072	0.334**	0.190**		0.423**	0.737**	0.668**	0.857**	0.772**
CHt	17	0.073**	0.297**	0.102**	0.177**		0.746**	0.618**	0.852**	0.763**
WNAC	29	0.345**	0.577**	0.355**	0.447**	0.355**		0.702**	0.852**	0.840**
GM	14	0.103**	0.339**	0.132**	0.210**	0.111**	0.394**		0.857**	0.739**
SA	38	0.446**	0.676**	0.447**	0.546**	0.458**	0.648**	0.501**		0.867**
CHa	19	0.091**	0.312**	0.120**	0.194**	0.099**	0.366**	0.129**	0.465**	

Nucleotide divergence (Da; Nei 1987) between populations was computed and used to reconstruct a Neighbor-Joining tree comparing the populations that were analysed by both microsatellite DNA and mtDNA markers (Fig. 2). The mtDNA and microsatellite DNA trees were broadly congruent with 12 out of 15 quartets of the same type resolved in both trees, with the remaining three quartets resolved in both trees, but of different types (after Estabrook, 1992). In a separate analysis of distance measures comparing all *T. truncatus* with *T. aduncus* and *D. delphis*, the SA *aduncus*-type population was at least as differentiated from the Chinese *aduncus*-type population (Da=0.035) as from the *truncatus*-type populations (Da=0.019), and was least differentiated from *D. delphis* (Da=0.013). A parallel assessment using the microsatellite data (see above) gave a Da of 0.438 between *T. truncatus* and *T. aduncus* (SA), 0.267 between *T. truncatus* and *D. delphis*, and 0.541 between *T. aduncus* (SA) and *D. delphis*.

The spanning network (Fig. 4) reflects the diverse genotypes found among the WNAP and MS samples, and suggests a relatively stable population structure for these regions, given the occurrence of these population genotypes in multiple clusters. The clusters representing GM, WNAC and BAH, on the other hand, reflect the reduced variation seen in those populations and suggest local demographic

expansions. The three haplotypes found in the ENA sample (MS1, ENA1 & ENA2) all fall within the central cluster, but are not closely related to each other.
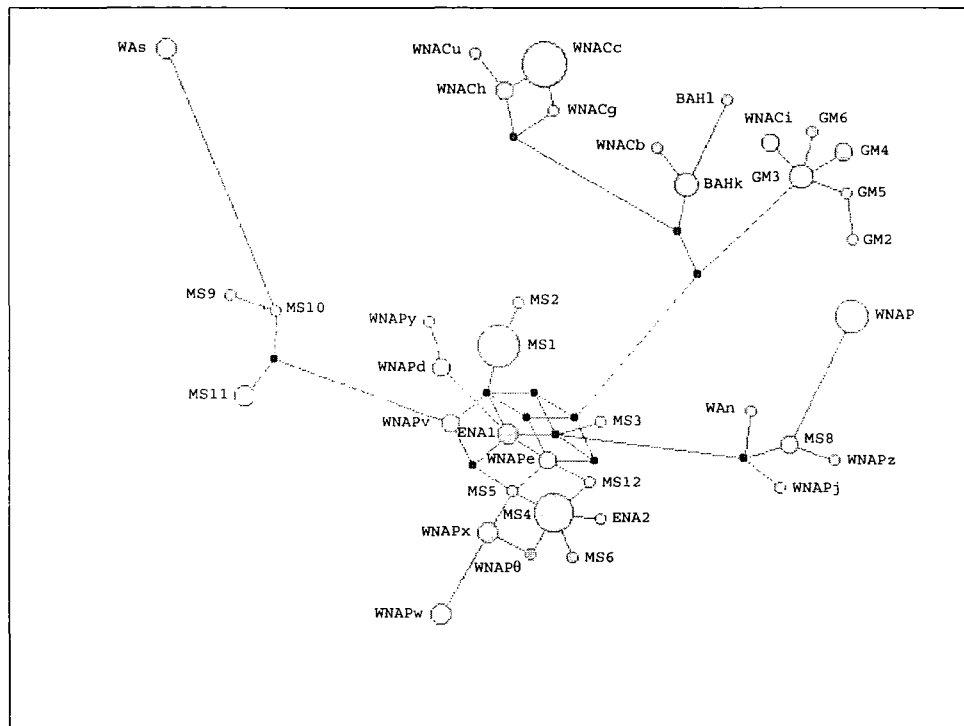
Figure 4: Minimum spanning network of the Atlantic and Mediterranean *T. truncatus* haplotypes only. Names of the haplotypes are the same as in Fig. 3. Black circles indicate ancestral extinct haplotypes.

Rooted (*Orcinus orca*) neighbour-joining and maximum parsimony trees were reconstructed using all 66 different mtDNA haplotypes (Fig. 5).
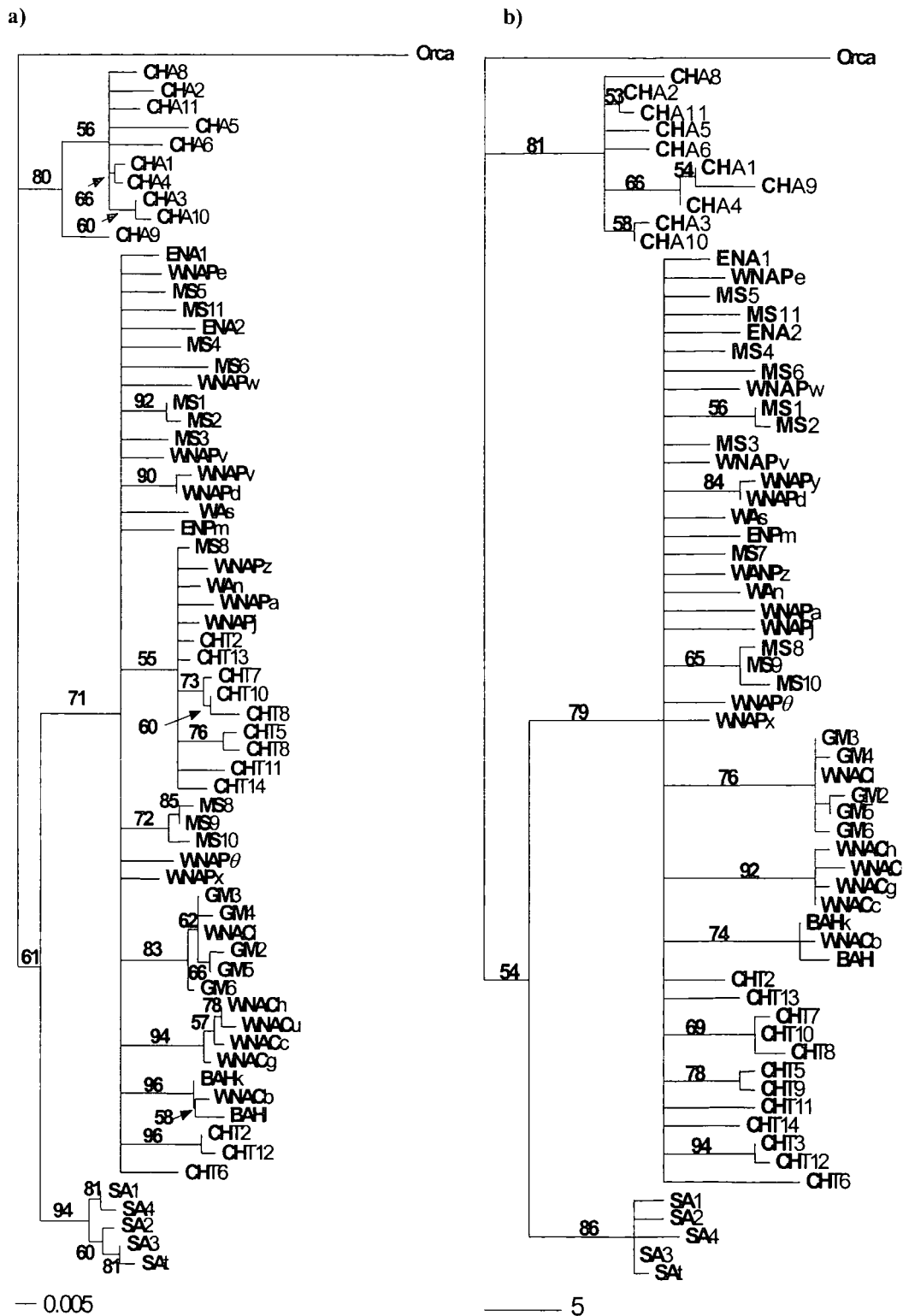
Figure 5: Neighbor-Joining (a) and Maximum parsimony (b) trees illustrating the phylogenetic relationships among 66 mtDNA haplotypes (names correspond to those given in Fig. 2, and the abbreviations in bold refer to the geographic source of the sample). Bootstrap values greater than 50% are indicated. *Aduncus* type haplotypes are represented by SA (South African *aduncus*-type) and CHa (Chinese *aduncus*-type). Chinese *truncatus*-type samples are represented by CHt.

Both *aduncus*-type populations (SA and CHa) were differentiated from the *truncatus*-type samples, but the two *aduncus*-type populations also strongly diverged from each other. Haplotypes from WNAC, GM and BAH form three well-supported lineages within the broader *truncatus*-type lineage, which is otherwise dominated by the pelagic samples. Both WNAC and GM populations each have one relatively common haplotype (WNACc and GM3, respectively; see Fig. 3 & 4), and several other less common haplotypes that differ by one or two base pairs from the common haplotype. Average genetic distance between these lineages was 2.91% (± 0.67%; one s.d.), compared to 0.55% (± 0.28%) within lineages. The exceptions are WNACi, which clusters closely with the GM haplotypes, and WNACb, which clusters with the BAH haplotypes. Another lineage, relatively poorly supported (55%) within the broader *truncatus*-type lineage (but only for the neighbour joining tree; Fig. 5) included a mixture of haplotypes from several regions including many of the Chinese *truncatus*-type haplotypes.

Discussion

Throughout the geographic regions included in this study, the genus *Tursiops* shows considerable genetic diversity and differentiation among populations. In fact, all putative populations defined by geographic region or habitat use (such as coastal and pelagic populations in the western North Atlantic), showed private alleles and significant differentiation from all other putative populations at both mtDNA and microsatellite DNA loci (Fig. 3 & Table 2). Marine mammal species are highly mobile and capable of long-range dispersion (see review in Stevick *et al.*, 2002). Often population structure is more evident for mtDNA markers than for nuclear DNA for marine mammals (see review in Hoelzel *et al.*, 2002), and in some cases this is likely due to greater dispersal by males (e.g. for the southern elephant seal: Slade *et al.*, 1998, Fabiani *et al.*, 2003). However, for the bottlenose dolphin, gene flow seems to be reduced among populations for both sexes. The large values for F_{ST} based on microsatellite data, and the strong correlation between F_{ST} values based on microsatellite and mtDNA data supports this interpretation. Bottlenose dolphin social groups are relatively fluid, however there is also some indication from observational data that neither males nor females disperse far from their natal groups (Scott *et al.*, 1990).

Phylogenetic reconstructions support earlier suggestions for the classification of the Chinese *aduncus* morphotype as an ESU (Wang *et al.*, 1999), but also suggest that the South African coastal population of '*aduncus*-type' dolphins represents an independent lineage from both the *truncatus*-type populations and '*T. aduncus*' from China. The high distance values for microsatellite DNA markers also indicate substantial differentiation between the South African *aduncus*-type and all other populations. Since the initial description of the '*aduncus*' form was based on the South African population (Ross 1977), we propose (given that further data continue to support the interpretation of isolation and differentiation among these populations) that this 'species' retains the name *T. aduncus*, while the Chinese population could be re-classified as a third species. However, the inclusion of *Delphinus delphis* in our distance comparisons, and the closeness of especially the South African *aduncus* form to this species, raises the issue of generic classification as well (as earlier indicated in LeDuc *et al.*, 1999). We have not, however, attempted any further resolution of the generic status of these species in this study. Both the neighbor joining and the maximum parsimony phylogenetic reconstructions supported the same lineage structure for the *T. truncatus* populations, with the exception of a lineage with 55% bootstrap support in the neighbor-joining tree, not supported in the maximum parsimony tree. This lineage was dominated by pelagic samples. Lineages representing coastal populations (WNAC, GM and BAH) were well supported in both reconstructions.

The lack of significant differentiation in microsatellite DNA allele size among populations suggests that genetic drift is important and that the rate of gene flow may be high relative to the mutation rate. For the mtDNA data, F_{ST} and Φ_{ST} were similar for comparisons among populations from the eastern North Atlantic, pelagic western North Atlantic, West Africa and the Mediterranean, but Φ_{ST} values were much larger for some other comparisons (Table 4). This was especially true for comparisons between the aduncus-type populations and the rest, and for the coastal populations in the western North Atlantic in comparison with the eastern North Atlantic and pelagic populations. The implication is that there has been greater time for sequence divergence among these populations.

Genetic diversity was highest for the population samples known to be from pelagic sources (WNAP for both mtDNA and microsatellite markers, and CHt based on published data for mtDNA diversity). The coastal populations mostly showed

lower genetic variation, including significantly lower allelic richness and heterozygosity (which were also very consistent among the coastal populations; Table 2). This could reflect independent historical founder events, with pelagic populations representing the source. For example, WNAP may represent a source population for founders establishing GM, BAH and WNAC populations in the western North Atlantic, though this would suggest that the founder genotypes were rare in the source population, and either unsampled or extinct in the current pelagic population (for further discussion see Hoelzel *et al.*, 1998a). The South African *aduncus* population may also have been founded from an unidentified source population, though as suggested above, the taxonomic issue has yet to be resolved. Historical bottlenecks in coastal populations, or demographic cycles could also account for reduced diversity.

While the spanning network (Fig. 4) reinforces the interpretation of founder origins for WNAC, GM and BAH, for ENA shows a more complex structure. This could suggest multiple founder events or a source population not well represented in our sample. Although coastal, the MS population shows nearly as much diversity as WNAP, but is also the least differentiated from WNAP, suggesting recent or continuing gene flow. The samples from MS may also be somewhat heterogeneous, as several geographic areas within the Mediterranean basin are represented. The significant deficiency (compared to H-W expectations) of heterozygotes at two microsatellite DNA loci in MS may therefore reflect some population structure within this sample (Wahlund effect).

Differential social structure in the coastal and pelagic populations is a possible alternative explanation for the difference in diversity, but there are no data to support this, and the observed pattern of diversity in at least some of the coastal populations is more consistent with founder events. One possible mechanism for the establishment of coastal founder populations would be the release of suitable habitat during interglacial periods. A recent study on harbor porpoise (*Phocoena phocoena*) phylogeography in the North Atlantic suggested an influence of the last glacial epoch on their distribution and population genetic structure (Tolley *et al.*, 2001).

The pattern of mtDNA variation among samples from the Gulf of Mexico, the Bahamas and the WNA coastal region suggest demographic events that left one dominant matriline at each location. In the WNA coastal population however, two haplotypes (represented by three individuals) stand out as highly differentiated

compared to other samples from that region. One of these haplotypes falls clearly into the GM lineage, and the other into the BAH lineage (Fig. 4). These could represent female dispersal events from GM and BAH populations into the WNAC population.

The high level of differentiation among regional populations suggests a high potential for speciation in this genus. Several of the *T. truncatus* populations show reduced variation and a pattern of variation consistent with population expansion. A possible scenario to explain this pattern could be that peripheral populations might have formed as founders at different times in the past from relatively large and diverse pelagic populations. While we have not fully characterized the putative pelagic populations, relatively low diversity, evidence for expansion in Tajima's D, and the structure of the spanning network reconstruction support this interpretation for at least some coastal populations.

Population structure in marine vertebrates can range from relative panmixia (e.g. the European eel, *Anguilla anguilla*, Daemen *et al.*, 2001) to highly structured populations for species with limited dispersal range (e.g. *Acanthochromis polycanthus*, Planes *et al.*, 2001). Atlantic and Pacific populations of striped mullet (*Mugil cephalus*) were highly differentiated (Rossi *et al.*, 1998) while several tuna species are not differentiated among oceans (Graves, 1996). Differences among species may in some cases be due to life history characteristics. For example, the relatively sedentary common sole (*Solea vulgaris*) shows population structure in the Mediterranean Sea (Guarniero *et al.*, 2002), while the highly mobile swordfish (*Xiphias gladius*) apparently does not (Pujolar *et al.*, 2002). At the same time, closely related species with similar life histories may show very different patterns of population structure (e.g. comparing *Dicentrarchus labrax* and *D. punctatus*, Bonhomme *et al.*, 2002). It seems most likely that there will typically be multiple factors involved. For example, Riginos and Nachman (2001) found extensive population structure for a small subtidal reef fish (*Axoclinus nigricaudus*) in California, and concluded that this structure was due to a combination of biogeography, geographical distance and the availability of suitable habitat. In our study on bottlenose dolphins we found a high degree of population structure among geographic regions, including differentiation between parapatric populations that share the same coastal habitat (WNAC and GM), and differentiation between three apparent ESUs, *T. truncatus* and the two *aduncus*-types in South Africa and China.

The data suggest a combination of factors leading to population structure, including the utilization of different local habitats, and possibly historical factors leading to the founding of new populations.

Acknowledgements

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CHAPTER 3

Habitat structure and the dispersion of male and female bottlenose dolphins (*Tursiops truncatus*)

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Abstract

Bottlenose dolphins (*Tursiops truncatus*) are widely distributed and a high degree of differentiation has been found among both allopatric and parapatric populations. We analysed 145 samples from the Black Sea, Mediterranean Sea and eastern North Atlantic for mitochondrial and nuclear genetic diversity, and found fine-scale population structure with boundaries that coincided with transitions between habitat regions. These regions were defined by bottom topography, and oceanographic features such as surface salinity and temperature. At the extremes of this range there was evidence for the directional emigration of females from marginal habitat. Bi-parentally inherited markers did not show this directional bias in migration, suggesting a different dispersal strategy for males and females at range margins. However, comparative assessment based on mtDNA and nuclear markers suggested that neither sex showed greater dispersal on average. These data suggest a mechanism for the evolutionary structuring of populations based on local habitat dependence for both males and females of this species.

Introduction

The Mediterranean Sea and the Black Sea represent a unique marine ecosystem. Geographically they consist of a sequence of contiguous basins, separated from the Atlantic Ocean by a narrow strait at Gibraltar. Both the Strait of Gibraltar and the straits that connect the Mediterranean and Black Sea basins (the Turkish Strait System) have been suggested to represent a barrier to gene flow for some species (e.g. *Merluccius merluccius*, Roldàn *et al.*, 1998; *Sardinella aurita*, Chikhi *et al.*, 1997; *Sepia officinalis*, Perez-Losada *et al.*, 2002; *Balaenoptera physalus*, Bérubé *et al.*, 1998; *Stenella cereuoalba*, Garcia-Martinez *et al.*, 1999). For some species investigated on a finer geographic scale, the most likely boundary was identified as not the Strait of Gibraltar, but an oceanic front some 350km to the east: the Almeria-Oran oceanic front, where the Atlantic oceanic waters encounter the warmer and denser Mediterranean waters (e.g. *Dicentrarchus labrax,* Naciri *et al.*, 1999; *Mytilus galloprovincialis,* Quesada *et al.*, 1995). However, any structuring across these putative boundaries would have to have been recent, as the shape and connectivity of these basins has changed considerably over the course of the Holocene, and as recently as 7,900 years ago (e.g. Ryan *et al.*, 1997).

The Mediterranean Sea and Black Sea offer a wide variety of different oceanographic environments, ranging from very shallow waters and sandy floors in the Adriatic Sea, to very deep abyssal areas in the Ionian Sea, and oceanographic discontinuities can be identified throughout the whole range. The Mediterranean Sea is generally characterised by higher salinity and higher water temperature compared to the Atlantic Ocean. On the other hand, the Black Sea is characterised both by low salinity, due to high outflow of fresh water from rivers, and low water temperature, especially during the winter season when the water usually freezes in the northeast (e.g. in the Azov Sea).

Bottlenose dolphins are observed throughout the geographic range of our study from Scotland to the Black Sea. In both inland seas they commonly inhabit coastal areas (Notarbartolo di Sciara *et al.*, 1993), although occasional sightings offshore and long-distance movements have been reported (Morozova, 1981; Derhmain *et al.*, 1999). The Black Sea bottlenose dolphin is considered an endemic subspecies named *Tursiops truncatus ponticus*, based on morphological data that show a significant reduction of body size when compared to bottlenose dolphins from other areas (Tomilin, 1957; Hershkovitz, 1966). The same situation has been observed for

the other two cetacean species inhabiting the Black Sea: the common dolphin and the harbour porpoise, and they too are considered different subspecies (*Delphinus delphis ponticus* and *Phocoena phocoena relicta*; Tomilin, 1957; Hershkovitz, 1966).

The bottlenose dolphin shows strong population genetic structure across its worldwide range (Chapter 2), not always correlated to geographic distance. For a highly mobile species such as the bottlenose dolphin, able to migrate for long distances (Wells *et al.*, 1999, Wood 1998), such extensive structuring of populations was not expected. Our hypothesis is that the capacity to adapt to local environments combined with a dependence on social behaviour for resource exploitation has led to the fine-scale population structure seen in coastal populations of this species, and that this may also explain the evolution of population structure in similar species. Here we compare contiguous populations across a geographic range that represents clear habitat structure, and assess the strength and position of population genetic structure, and patterns of gene flow in this context.

Materials and Methods

Sample collection and DNA extraction

Samples were collected from stranded animals, by biopsy sampling or scrub sampling (sloughing skin collected on plastic scrub pads). DNA was extracted from tissue samples preserved in salt saturated 20%DMSO by a standard phenol/chloroform extraction method (Hoelzel, 1998).

A total of 145 samples were included (Fig. 1). Of these, 81 samples were analysed in this study for the first time (16 from the Black Sea & Crimea, 2 from the Ionian Sea, 3 from the eastern north Adriatic, 26 from Spain, 5 from the Balearic Islands, 11 from Portugal, and 18 from Galicia), and they were compared with previously analysed samples (3 from Israel, 7 from the Ionian sea, 8 from eastern north Adriatic, 9 from the western Adriatic Sea, 10 from the Tyrrenian Sea, 1 from Algeria, 6 from South England and 20 from Scotland; from Chapter 2) for the same loci.
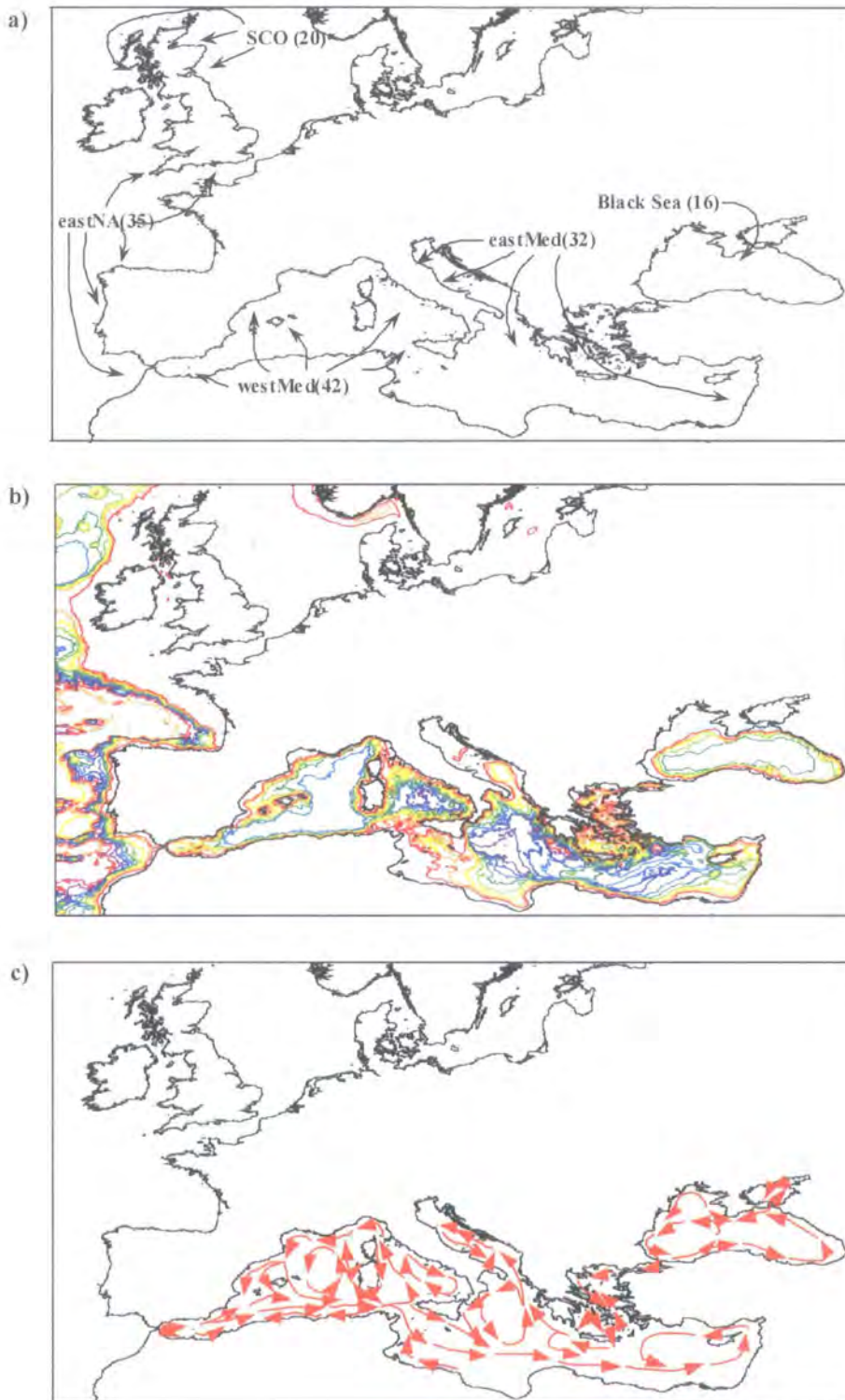
Figure 1. a) Map of the sample locations. Abbreviations are as follows: eastMed = eastern Mediterranean, westMed = western Mediterranean, eastNA = eastern North Atlantic, Sco = Scotland. **b)** A map showing the depth profile and **c)** the current pattern in the Mediterranean Sea are also reported.

Sex determination

Individuals whose gender was unknown were sexed by amplifying portions of the genes ZFX and ZFY as described in Bérubé and Palsbøll (1996).

Microsatellite analysis

Samples were genotyped at 9 microsatellites loci: KWM1b, KWM2a, KWM2b, KWM9b, KWM12a derived from *Orcinus orca* (Hoelzel *et al.*, 1998b), EV37Mn from *Megaptera novaeangliae* (Valsecchi & Amos, 1996), TexVet5, TexVet7 and D08 from *Tursiops truncatus* (Rooney *et al.*, 1999, Shinohara *et al.*, 1997). PCR conditions were as reported in Chapter 2. Amplified DNA was analysed for length variation on 6% polyacrylammide denaturing gels using fluorescent imagining on an automated ABI PRISM 377 DNA sequencer, after incorporation of 1/10 fluorescent labelled primer. An internal standard marker (Genescan-500 ROX, Applied Biosystems) was used to determine the allele sizes.

The level of genetic diversity was estimated as observed heterozygosity (H_o), expected heterozygosity (H_e), and allelic richness. Allelic richness controls for variation in sample size by a rarefaction method, and was calculated using the program FSTAT 2.9.3 (Goudet, 2001). Evaluation of possible deviations from Hardy Weinberg (overall deviation, heterozygote deficiency and heterozygote excess) was performed using Fisher's exact test and the Markov chain method (dememorization number, number of batches, iteration per batch set at 1,000, Bonferroni correction applied) using GENEPOP 3.1d (Raymond & Rousset, 1995a,b).

The most probable number of putative populations (K) that best explains the pattern of genetic variability was estimated using the program STRUCTURE 1.0 (Pritchard *et al.*, 2000). We assumed the admixture model and performed the analysis considering both the independent and the correlated allele frequency model. Burn in length and length of simulation were set at 1,000,000 repetitions. To test the convergence of the priors and the appropriateness of the chosen burn in length and simulation length, we ran a series of independent runs for each value of K (for $1<K>8$) as suggested by Pritchard *et al.* (2000). We tested whether any particular individual was an immigrant or had an immigrant ancestor by using the model with prior population information, subdividing the individuals into K populations, according to the results of the previous analysis. We assumed *v* (migration rate) = 0.05 and 0.1, and testing for $0<$number of generations (G)>1.

An asymmetric estimate of the migration rate ($M=4N_em$) between pairwise populations, based on microsatellite and mtDNA data, was calculated using MIGRATE 1.7.3 (Beerli, 2002). The lengths of the runs were optimised for both markers (acceptance-rejection > 2%, R<1.2). Initial runs were set estimating θ and M with F_{ST} and allowing M to be asymmetric. Reruns were set using the parameter estimated found with the first run and lengthening the MCMC chains. In order to verify the result a final run was set using longer chains. For comparison the migration rate was also calculated from F_{ST} according to $F_{ST}=1/(4Nm+1)$.

Genetic distances between populations and between individuals were estimated using Nei's Da genetic distance (Nei *et al.*, 1983). Calculations were performed using the programme MicroSatellite Analyser (MSA) (Dieringer and Schlotterer, 2002).

Multidimensional scaling analysis was performed using the programme *XL-Stat Pro 6.0* based on a matrix of pairwise Da distances among individuals.

The level of differentiation among populations was estimated as F_{ST} (Weir & Cockerham, 1984) using the program ARLEQUIN 2.0 (Schneider *et al.*, 1999).

Sex-biased dispersal was tested using the program FSTAT 2.9.3 (Goudet, 2001). Only adult individuals (a total of 131: 61 females and 70 males) were considered for this analysis.

Evidence of a bottleneck was tested using the programme BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996). The Two-phase model of mutation (TPM) was considered as suggested by the authors (variance for TPM as set equal to 30, proportion of SMM in the TPM was set equal to 70%, 1000 iterations).

mtDNA analysis

A total of 99 samples were sequenced for 630 bps at the 5' end of the mtDNA control region (15 from Black sea, 18 from eastern Mediterranean, 31 from western Mediterranean, 35 from the eastern North Atlantic) and compared with 24 sequences already published (1 from Black Sea, 10 from eastern Mediterranean, 4 from western Mediterranean, 9 from Scotland, Chapter 2).

The mitochondrial DNA control region was amplified with universal primers MTCRf (5'-TTC CCC GGT GTA AAC C) and MTCRr (5'-ATT TTC AGT GTC TTG CTT T) after Hoelzel (1998). The PCR reaction conditions and PCR cycling profile were as reported in Chapter 2. PCR products were purified with QIAgen PCR

purification columns and sequenced directly using the ABI dye-terminator method. Sequence alignment was performed using SEQUENCHER 3.0 (Gene Code Corp.).

The degree of differentiation (F_{ST}), the nucleotide diversity (π), Tajima's D and Fu's F_S were estimated using ARLEQUIN 2.0 (Schneider *et al.*, 1999). The Mantel test to estimate the level of correlation between matrices was also performed using ARLEQUIN 2.0. A median-joining network was generated to infer phylogenetic relationships among the mtDNA haplotypes, using the program NETWORK 2.0 (Bandelt *et al.*, 1999; www.fluxus-engineering.com).

Results

Measures of diversity

Heterozygosity and allelic diversity for all nine microsatellite DNA loci are shown in Table 1. Deviation from HW equilibrium for $p<0.05$ was detected at one locus in the western Mediterranean population and at 4 loci in the eastern North Atlantic population. If Bonferroni correction is applied (new $p=0.0011$), no significant deviation was observed. Average allelic richness was lowest for the Black Sea population (3.639) and highest for the East North Atlantic population (6.207). Private alleles where found in all populations except the Black Sea.

Table 1. Genetic variation at each locus for each population. The number of individuals analysed for each population is indicated below the population name. The number of different alleles, number of private alleles (in parenthesis) and allelic richness (All. Rich.), heterozygosity observed (Ho), and heterozygosity expected (He) are reported. The respective averages (standard deviation in parenthesis) are reported in the last rows. The asterisk indicates the loci that showed significant deviation from the HW equilibrium (p<0.05). Abbreviations are as in Fig. 1.

Microsatellites		Populations				
		Black Sea N=16	eastMed N=32	westMed N=42	eastNA N=35	Scotland N=20
KWM1b	All. Rich.	2 2	3 (1) 1.988	2 1.998	2 1.822	2 1.943
	Ho	0.375	0.031*	0.286	0.114	0.05
	He	0.314	0.122	0.331	0.136	0.189
KWM2a	All. Rich.	3 3	5 4.914	6 4.646	6 5.582	4 3.593
	Ho	0.563	0.75	0.524	0.571*	0.75*
	He	0.675	0.756	0.605	0.674	0.639
KWM2b	All. Rich.	2 1.75	4 2.601	4 3.166	5 4.471	3 2.943
	Ho	0.063	0.156	0.341	0.371*	0.45
	He	0.123	0.179	0.406	0.581	0.522
KWM9b	All. Rich.	3 2.965	4 3.341	5 (1) 4.064	4 3.933	4 (1) 3.2
	Ho	0.4	0.419	0.525*	0.618	0.45
	He	0.402	0.48	0.68	0.667	0.582
KWM12a	All. Rich.	5 4.737	7 (1) 5.807	11 8.212	11(1) 9.083	7 5.399
	Ho	0.56	0.69	0.895	0.853	0.65
	He	0.677	0.734	0.863	0.896	0.68
EV37Mn	All. Rich.	7 7	15 (2) 11.781	23 (3) 13.241	19 (1) 12.834	9 7.22
	Ho	0.5	1	0.949	0.906	0.6
	He	0.562	0.928	0.929	0.926	0.778
TexVet5	All. Rich.	4 3.444	5 4.662	8 5.617	9 6.86	5 4.303
	Ho	0.25	0.533	0.568	0.552	0.722
	He	0.236	0.664	0.651	0.739	0.619
TexVet7	All. Rich.	4 3.887	4 3.242	7 (1) 4.375	6 (1) 5.089	4 3.2
	Ho	0.652	0.194*	0.595	0.543*	0.65
	He	0.53	0.341	0.592	0.68	0.53
D08	All. Rich.	4 3.965	6 5.448	6 5.742	8 (2) 6.192	4 3.833
	Ho	0.6	0.688	0.829	0.647*	0.5
	He	0.66	0.737	0.786	0.73	0.489
Average (SD)	All. Rich.	3.639 (1.574)	4.865 (2.904)	5.673 (3.333)	6.207 (3.195)	3.959 (1.550)
	Ho	0.464 (0.205)	0.549 (0.282)	0.649 (0.197)	0.670 (0.228)	0.557 (0.164)
	He	0.438 (0.186)	0.496 (0.321)	0.612 (0.234)	0.575 (0.237)	0.536 (0.213)

For the 630bp mtDNA control region sequence, forty-four polymorphic sites (7%) were observed identifying a total of 41 different haplotypes. Forty-two transitions, four transversions, and two indels were observed. The total average nucleotide diversity was 0.016. Average gene and nucleotide diversity was calculated for all populations. The Black Sea population showed the lowest gene diversity, whereas the Scottish population showed the lowest nucleotide diversity (Table 2).

Table 2. Genetic diversity (Gene Div.), nucleotide diversity (Nucl. Div.), and the values of tests for neutrality Tajima'D and Fu's Fs are reported for each population. Abbreviations are as in Fig. 1.

Pop	Gene Div.	Nucl. Div.	D	Fs
Black Sea	0.675	0.012	1.54	3.28
eastMed	0.88	0.015	0.16	0.14
westMed	0.939	0.013	-0.45	-1.68
eastNA	0.914	0.014	0.92	0.5
Scotland	0.694	0.006	-0.38	1.82

Inferring population structure

In order to test the presence of population structure among our samples, we used STRUCTURE (Pritchard *et al.*, 2001) to estimate the number of populations (K) that best explained the observed genetic variability. Consistency among different runs was observed for the estimate of P(X/K) and the prior α, indicating that the burn in length and the length of the runs were appropriate.

K=5 was found to be associated with the highest probability of P(X/K) considering either independent allele frequency or correlated allele frequency models suggesting subdivision into five populations (Fig. 2). The clustering identified three clear populations: a Black Sea population, an eastern Mediterranean population including samples from Israel, Ionian Sea, Adriatic Sea, and a Scottish population (Fig. 2). Two other putative populations: the western Mediterranean (Tyrrenian Sea, Spain, Algeria) and the contiguous eastern North Atlantic were less clearly defined (Fig. 2). In fact, the two clusters that explained most of the variability of the samples from these two regions (the third, q_3, and the fourth, q_4, – shown in Fig. 2 in yellow and green respectively) showed low allele frequency divergence (0.03). Therefore, in order to further assess possible population structure between the western Mediterranean Sea and the eastern North Atlantic, the number of populations (K) was estimated considering only the individuals from these regions, as suggested by Pritchard *et al.* (2000). However, no population structure was detected (the most probable number of populations found was for K=1). We then assessed this again using multidimensional scaling analysis, based on a D_A distance matrix among pairs of individuals. Clustering was observed consistent with subdivision between the individuals from the western Mediterranean and the eastern North Atlantic (Fig. 3).
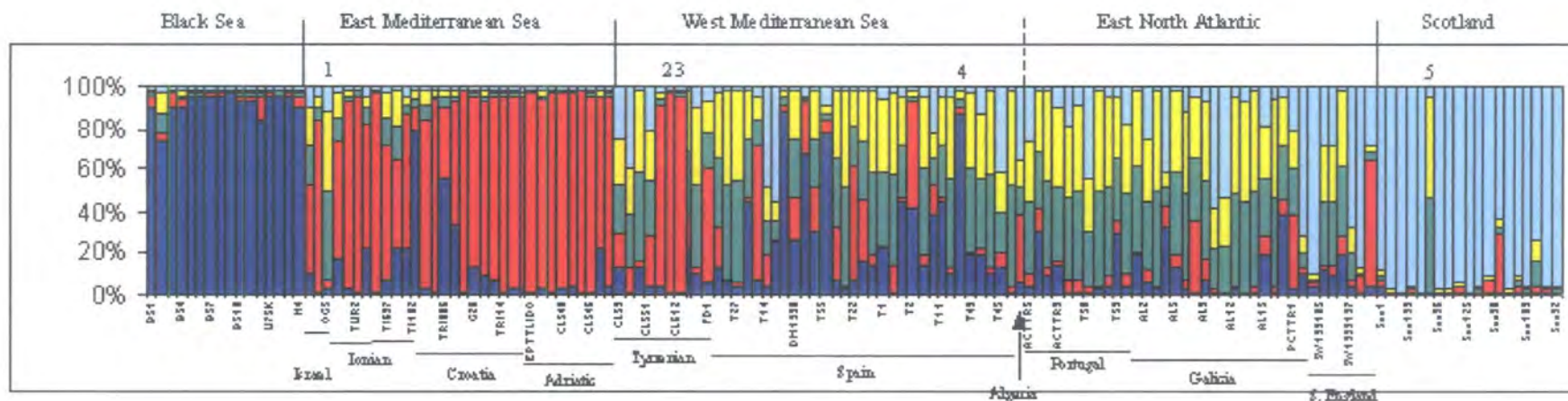
Figure 2. Estimated proportion of the coefficient of admixture of each individual's genome that originated from population *k*, for K=5. Each individual is represented by a column. Detailed geographic origin of the samples is reported below the graphic. The numbers 1-5 indicated the individuals identified as migrants (Table 3).
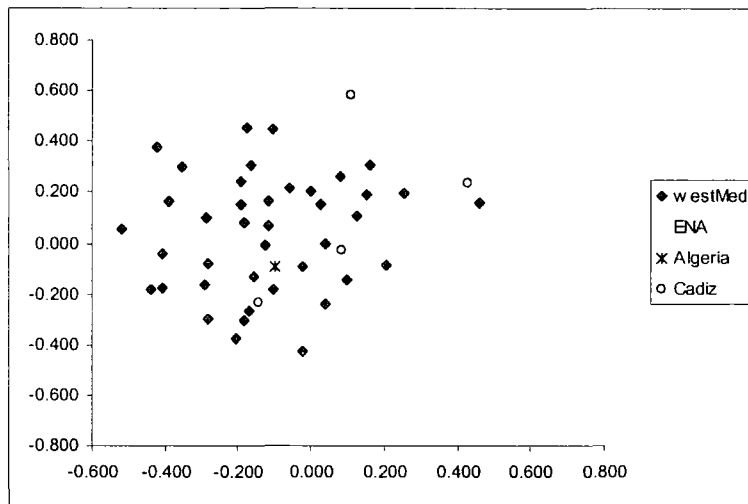
Figure 3. Multidimesional scaling analysis based on Da distance between pairwise individuals. Axes x and y represented dimension 1 and 2 respectively.

Returning to this region in the analysis in STRUCTURE where K=5, for each individual the admixture coefficients relative to the third and the fourth group were summed (q_3+q_4). The difference between the average of the individual sums (q_3+q_4) for the western Mediterranean individuals and eastern North Atlantic individuals was found to be significant (Mann-Whitney U-test, Z=1.962, p=0.024) indicating that the proportion of the ancestry coefficients (q_3+q_4) is different in the two groups of individuals considered.

Deviation from the Hardy-Weinberg equilibrium was tested for the pooled western Mediterranean/eastern North Atlantic group and significant deviation (p<0.05) was observed at 4 loci (one locus if Bonferroni correction was applied (p<0.0014)).

Differentiation among the five putative populations (Black Sea, eastern Mediterranean, western Mediterranean, eastern North Atlantic, and Scotland) at the microsatellite DNA loci was estimated as F_{ST} (Table 3). All populations were differentiated (p<0.001). The Black Sea population showed the highest level of differentiation when compared with all other populations. The western Mediterranean population was also differentiated from the eastern North Atlantic population, supporting the population subdivision suggested above.

Table 3. Pairwise population differentiation values expressed as F_{ST} based on microsatellite data (below diagonal) and mtDNA haplotype frequencies (above diagonal). Sample size for the microsatellites for each population is reported in the second coloumn. Sample size for the mtDNA data is reported in the second row. Statistic significance is reported as follows: * = p<0.05, ** = p<0.01, *** = p<0.001. Abbreviations are as in Fig. 1.

	N.	BlackSea	eastMed	westMed	eastNA	Scotland
N. haplo		16	27	35	35	9
BlackSea	16	–	0.041	0.093***	0.140***	0.317***
eastMed	32	0.120***	–	0.032**	0.058***	0.186***
westMed	42	0.102***	0.045***	–	0.040**	0.153***
eastNA	35	0.139***	0.081***	0.026***	–	0.076*
Scotland	20	0.211***	0.152***	0.097***	0.068***	–

For the mtDNA sequences, population differentiation was estimated as F_{ST} (Table 3). All pairwise population comparisons showed significant differentiation except for the Black Sea population compared to the eastern Mediterranean population (p=0.058). Significant correlation was found between the mtDNA and microsatellite DNA F_{ST} matrices (Mantel test, d.f.=4, p=0.02).

A median-joining network was drawn among the different mtDNA haplotypes to visualise the phylogenetic relationship (Fig. 4).
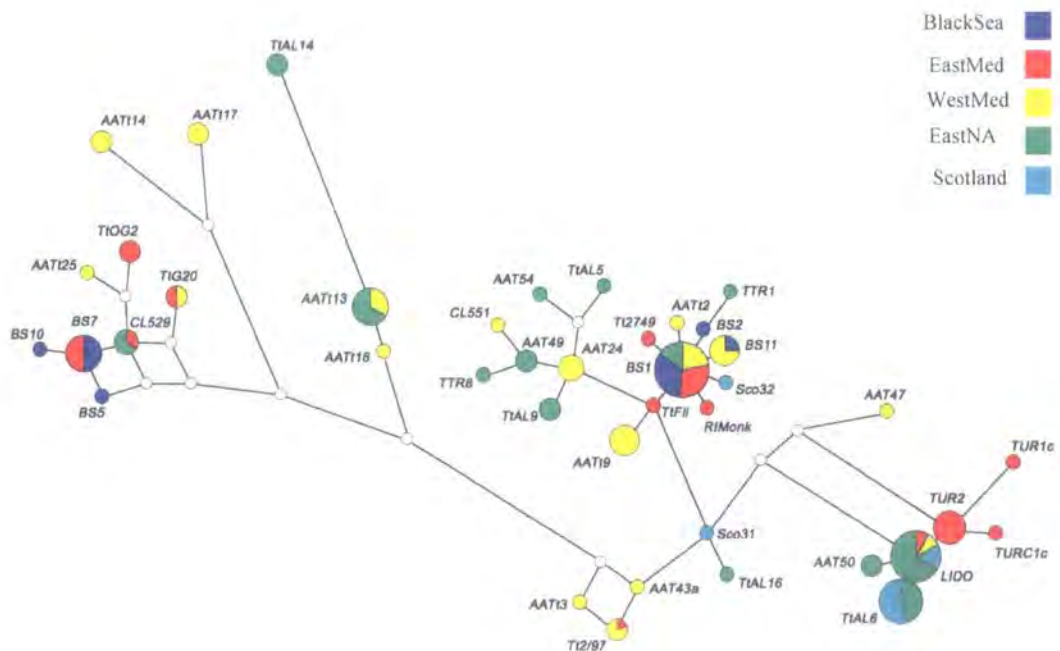
Figure 4. Median-joining network among haplotypes. The size of the circles is proportional to the total number of haplotypes observed. Sectors are proportional to the number of each haplotype observed in each population. Populations are identified as follows: Black Sea population in blue, EastMed population in red, WestMed population in yellow, EastNA in green and Scotland in light blue. White circles indicate ancestral extinct haplotypes.

Most of the highly represented haplotypes diverged consistently one from another identifying three main clusters. The Black Sea population is represented only in two clusters and all its unique haplotypes differ by one mutation step from the haplotype shared with the other populations. A similar situation is observed for the eastern Mediterranean population although it is represented in all three clusters.

No indication of recent bottleneck was observed for the Black Sea population (probability considering two tails for H excess or deficiency was 0.25).

A Mantel test showed a significant correlation between geographic and genetic distance for both mtDNA ($p=0.007$) and microsatellite DNA loci ($p=0.007$) (Fig. 5).
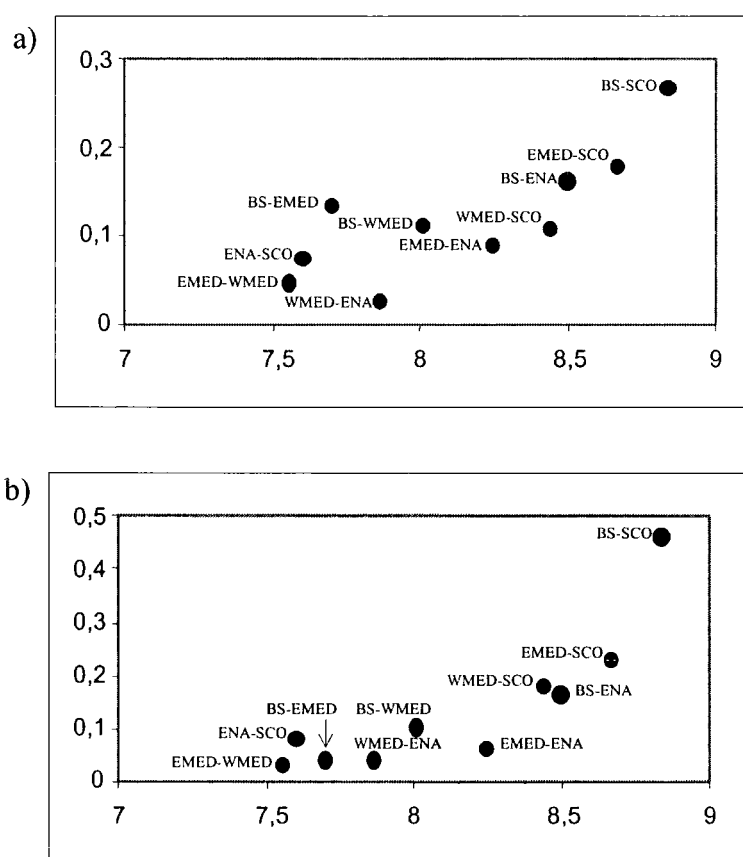
Figure 5. Geographic distance versus $Fst/(1-Fst)$ for pairwise populations based on a) microsatellite data, and b) mtDNA data. Geographic pairs are labelled (BS-Black Sea, EMED-eastern Mediterranean, WMED-western Mediterranean, ENA-eastern North Atlantic, SCO-Scotland).

Estimating migrants and sex-biased dispersal

We analysed whether individuals were possible immigrants or descendants of recent immigrants. Because no estimation of the coefficient of migration for the bottlenose dolphin was available in the literature, the analysis was performed three

times setting v to 0.001, 0.05 and 0.1 (as suggested in Pritchard *et al.*, 2000), and considering 4 populations. Five possible immigrant individuals were identified (four males and one female). All individuals were confirmed migrants at the values of v=0.1 and v=0.05, but none when v was set to 0.001. All individuals had higher probabilities to be immigrants rather than having immigrant ancestry. The Black Sea population did not show any immigrant individual from other areas, while one individual from the western Mediterranean was found to be a possible immigrant from the Black Sea population. In the eastern Mediterranean population the only immigrant individual detected was one of the three samples from Israel. In the western Mediterranean population two possible immigrants from the eastern Mediterranean population were found. Among the Scottish samples one individual was found to be a possible migrant from the western Mediterranean or eastern North Atlantic populations.

We estimated the migration rate (M) between contiguous populations using two different methods and the results are reported in Table 4.

Table 4. Estimate of the migration rate (M) between contiguous populations, based on the microsatellite and mtDNA data. Nm columns refer to the values calculated according $F_{ST}=1/(4N_em+1)$. The other columns refer to the asymmetrical migration rate calculated using a maximum likelihood method (MIGRATE): 1,2 stands for: migration from population 1 to population two; 2,1 stands for: migration from population 2 to population 1. The interval confidence (95% c.i.) is also reported. Abbreviations are as in Fig. 1.

Population	microsatellites (bi-parental)				mtDNA (maternally inherited)			
	Nm	1,2	2,1	95% c.i.	Nm	1,2	2,1	95%c.i.
1 BlackSea	1.830	--	3.490	3.065-3.986	11.690	--	0.046	0.04-0.415
2 east Med		3.076	--	2.714-3.47		13.304	--	3.986-53.572
1 east Med	5.306	--	7.813	7.331-8.326	15.125	--	0.558	0.495-2.322
2 west Med		12.857	--	11.935-13.837		10.074	--	4.711-20.845
1 west Med	9.365	--	13.610	12.87-14.382	12.000	--	4.560	4.524-12.608
2 east NA		16.630	--	15.691-17.652		5.349	--	1.092-5.816
1 east NA	3.426	--	6.791	6.17-7.45	6.079	--	20.373	13.719-26.546
2 Scotland		4.892	--	4.427-5.376		3.036	--	2.010-4.253

Sex-biased dispersal was tested and there was no indication of sex biased dispersal. In fact no significant heterozygosity deficiency or positive F_{IS} were observed for either sex (F_{IS} for females was 0.079, F_{IS} for males was 0.028, p=0.18), and the assignment index was not significant (p=0.73, assignment index variance: p =0.54).

Discussion

We find clear population structure over a small geographic range for contiguous populations of the bottlenose dolphin. The putative population boundaries were identified on the basis of comparing individual genotypes in the context of equilibrium expectations with respect to Hardy-Weinberg and linkage (see Pritchard *et al.*, 2000). The result was the assignment of population boundaries that correspond to physical boundaries in the environment, but none of these are likely to actually restrict the movement of bottlenose dolphins. Instead, they seem to define different habitat regions. Three of the boundaries are relatively strong, suggesting low gene flow, while a fourth is much less well defined.

To take them in turn, the first boundary separates a population in Scotland from samples further south in the North Atlantic. The estimated level of gene flow is relatively low between these two populations, and the mtDNA data suggest a higher rate of emigration than immigration for the Scottish population. One factor could be geographic distance, as the sample sites are separated by approximately 1200km. However, Scotland is at the extreme range limit of this species and this is likely to define the habitat for this population with respect to prey resource and related factors. Being at the range limit may also mean that this habitat is marginal with respect to its capacity to support the population. This would be consistent with the suggested history of emigration based on the mtDNA coalescent data. Photo-identification studies have reported a relatively high degree of residency among the bottlenose dolphins inhabiting this part of Scotland (Wilson *et al.*, 1999), and the level of genetic diversity is comparatively low.

The next boundary divides the North Atlantic samples (collected from Galicia and Portugal) from the western Mediterranean Sea. This is the weakest of the four boundaries, suggesting continuing gene flow or a very recent division. The strait of Gibraltar provides a physical boundary, but not one that is likely to restrict the movement of dolphins or their prey. However, the oceanographic feature at the eastern end of the Alboran Sea, the Almeria-Oran front, may serve as a barrier to the movement of some prey species, and perhaps in this way define local populations of their predators. For example, cuttlefish (*Sepia officinalis*; Perez-Losada *et al.*, 2002) and sea bass (*Dicentrachus labrax*; Naciri *et al.*, 1999) both show differentiation either side of this front. If this is the case, it may be recent or a weak mechanism for the isolation of bottlenose dolphin populations, as the data suggest relatively high, bi-

directional rates of gene flow across this boundary. Of the five samples collected nearest to the Almeria-Oran frontal region, four were near a boundary region in the MDS plot (Fig. 3).

The next boundary is again stronger, representing the western and eastern basins of the Mediterranean Sea, separated by the Italian peninsula. Differentiation between the eastern and the western Mediterranean has also been observed in other marine species like the common sole (*Solea vulgaris*; Guarniero *et al.*, 2002) and the sea bass (*Dicentrarchus labrax*; Bahri-Sfar *et al.*, 2000). In those studies the authors suggested that differences in hydrographic characteristics defined the different habitats in these two basins, and promoted the differentiation of intraspecific populations. While the western Mediterranean is more influenced by the Atlantic Ocean, the eastern Mediterranean is characterised by water circulation limited to the Libico-Tunisian Gulf, and by low activity in the rest of the basin (the Adriatic and Aegean Seas), which is under the influence of cool and low salinity waters (Pinardi *et al.*, 1997). Again, differences in the distribution of prey, reflecting differences in habitat, may be defining the geographic range and patterns of association in local populations of the bottlenose dolphin.

The final boundary is perhaps the strongest, separating the Mediterranean and Black Seas. Oceanographic conditions change quite dramatically across this boundary, with surface salinity and temperature both very different in the two seas. However, there are also potential historical factors, suggesting a possible founder event when the Strait opened, approximately 7,800 years ago. Consistent with this is the comparatively low level of diversity found in the Black Sea sample, and the lack of private alleles. However, various tests for evidence of a bottleneck showed no indication of one. This could be due to low power as a consequence of the small sample size, or may instead indicate that the diversity is low because the effective size of the Black Sea population is relatively small. Data from Migrate showed the strongest directional effect for gene flow in this population, again suggesting the emigration of females from peripheral (possibly marginal) habitat.

Taken together these data suggest that local populations of bottlenose dolphins are habitat dependent in a way that defines patterns of movement. A comparative assessment of estimates of gene flow for mtDNA and bi-parental markers, together with the very similar pattern of F_{ST} values for the two marker types, indicate that this pattern of movement is true for both sexes (with the exception of differential female

movement at range margins). The most likely mechanism seems to be social facilitation of foraging strategies within local communities of dolphins, tending to keep both males and females near their natal site. Transferable knowledge over generations could be advantageous to assure feeding success, however it implies complex social structure and long-term individual associations across generations (see Whitehead, 1998). This could lead to fine-scale structure at the intra-specific level, and could possibly lead to relatively frequent speciation within the genus (see Chapter 2). However, as indicated by the mtDNA spanning network data, structuring across the study range is likely quite recent, as there is no indication of lineage sorting. Furthermore, coastal habitat can be ephemeral, and so the population structure may change with environmental change in future.

Acknowledgements

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CHAPTER 4

Population structure of bottlenose dolphins impacted by bycatch along the east coast of South Africa

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Abstract

The state of the bottlenose dolphin (*Tursiops aduncus*) population inhabiting the waters of KwaZulu-Natal (South Africa) is an increasing cause for concern. Shark nets placed along the coast to protect bathers from shark attacks result in an incidental by-catch of dolphins twice as high as that suggested as the maximum sustainable capture rate of a cetacean population. Observational data reported the presence of two populations, a coastal population and a seasonal migratory population moving into the KwaZulu-Natal area following the movement of sardines.

We analysed nine microsatellite loci and 599bps of the mitochondrial control region from 142 samples of entrapped and free ranging individuals, and found small but significant differentiation between the northern and southern coastal populations. However, no differentiation was found between the southern coastal and putative migratory population. Genetic diversity was low for both marker types, and the pattern of mtDNA variation was consistent with a founder event. These results are discussed in the context of conservation and management.

Keywords: South Africa, bottlenose dolphin, *Tursiops aduncus*, population genetics, conservation.

Introduction

The continued by-catch of bottlenose dolphins (*Tursiops aduncus*) in the shark nets placed along the KwaZulu-Natal coast (KZN) of South Africa is a cause for concern (Peddemors *et al.*, 2002). Survey studies estimated the maximum size of the bottlenose dolphin population inhabiting this area to be around 870 individuals (Cockcroft *et al.*, 1991; Cockcroft *et al.*, 1992), and a comparison of sighting rates collected over 14 years, reported a drastic decline of the monthly sightings of this species (Cockcroft *et al.*, 1990). Continuous by-catches in the shark nets could be one of the main factors that have lead to the decline of the population. Based on an eight year survey (1980-1988) (Cockcroft, 1990) the average number of incidentals catches observed reached 4% of the total estimated population, twice as high as the 2% considered a maximum sustainable capture rate for cetacean populations by the International Whaling Commission (Anon., 1991). Furthermore, a predominance of incidental catches of calves and mature females has been observed suggesting that the recovery of the population might be even more difficult (Cockcroft, 1990).

A recent worldwide phylogeographic and phylogenetic study on the bottlenose dolphin reported high isolation and differentiation of the South African population and proposed its classification as a different species. Furthermore, low genetic diversity at both nuclear and mitochondrial markers was detected, suggesting that this population may have gone through a recent bottleneck (Chapter 2).

The major biotic event that dominates the marine life of the area is the annual winter migration of sardines *Sardinops ocellatus* (June through October) from the southern Cape into KZN waters, known locally as the 'sardine run'. The movement of these large shoals of fish is followed by thousands of predators including bottlenose dolphins. An investigation of the incidental capture of dolphins in the shark nets shows a seasonal pattern with increased catches during the 'sardine run period' (SRP), and for the bottlenose dolphin this is true especially along the South coast of KZN (Cockcroft, 1990).

The bottlenose dolphins occur in KZN waters all year round. Survey studies suggested the existence of two populations of bottlenose dolphins converging in the area: a 'resident' population occurring in the area all year round and a 'migratory' population occurring in the area only during the winter months, and coinciding with the movement of the sardines. The population recognised as 'resident' forms mainly small groups and is found in the coastal waters (within 10 km from the shore).

Estimates of the size of this population stand at 520 (160-970, 95% confidence limit) individuals for the North KZN coast (Cockcroft *et al.*, 1992), and a maximum of 350 for the South KZN coast (Cockcroft *et al.*, 1991). By comparison the 'migratory' population occurs in the KZN waters only seasonally. It is characterised by large schools of hundreds of individuals coming from at least as far south as Plettenburg Bay (South Eastern Cape) and moving north into the KZN waters in pursuit of the fish. These groups are not observed further north than Ifafa (Fig.1) and the size of this population is estimated to be over 2000 individuals (Peddemors, unpublished data). The seasonal occurrence of the migratory population during winter and the predominant summer-breeding period of this species suggest that these two populations might not interbreed.
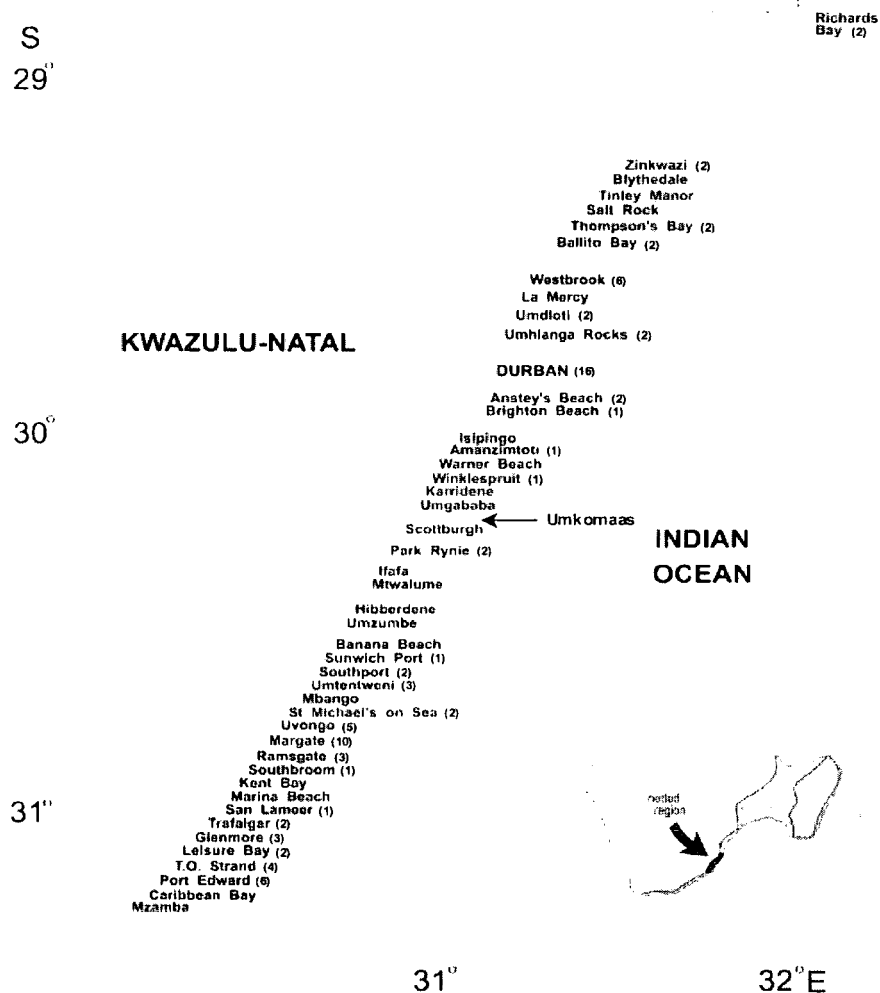
Figure 1. KwaZulu-Natal coast. All the shark nets stations are reported. In brackets the number of samples analysed from each stations are reported.

Analysis of the coastal distribution patterns suggested the subdivision of the resident population into small groups each inhabiting 'preferred' home range areas along the coast characterised by a higher dolphin frequency (Cockcroft *et al.*, 1990). Different studies have been carried out to shed light on this population structure. Analysis of organochlorine levels in individuals incidentally caught in the shark nets along the Natal coasts showed a regional variation of the pollutant burden, with a decline from north to south suggesting discreteness of the different groups (Cockcroft *et al.*, 1989). However, little variation was observed between dolphins captured during and outside the sardine run period. A preliminary genetic study based on 3 allozyme loci and 40 samples of dolphins caught in the nets in different preferred areas, also suggested possible genetic differentiation among subgroups inhabiting the north and south coasts of KZN. However, no significant differentiation was found when comparing North versus South (Goodwin *et al.*, 1996).

In both these studies the weak structure observed (between dolphins caught during and outside the SRP, and between the North and the South coast) could have been as a result of the presence in the South coast sample of individuals from both the resident and migratory populations.

A morphological character, the degree of ventral spotting observed in the by-caught individuals, was considered as possible method to distinguish between populations. However, no clear trend of this character was observed between individuals caught along the North and the South coasts, or between dolphins caught during the SRP and outside the SRP (Peddemors, unpublished data).

From all the data available, two different hypotheses of population structure along the KZN coasts seem plausible. In the first hypothesis a resident coastal population from north to south along the coast (possibly subdivided into discrete sub-populations) would be distinct from the seasonal migratory population. The coastal and migratory populations would overlap their habitat range along only the South coast and only for the period during the 'sardine run', but without effective interbreeding taking place. Alternatively a North coastal resident stock could be differentiated from a South coast resident-migratory stock. Genetic exchange would happen between the South coast resident population and the seasonal migratory population but less so with the northernmost resident population. In the first case the gene flow would be limited by a temporal factor, whereas in the second case the gene flow would be limited by a geographic factor.

In this study our objective is to assess the population structure of the bottlenose dolphins along the KZN coast in order to test these hypotheses and provide useful information for the implementation of effective conservation measures.

Materials and Methods

Samples origins

A total of 142 samples were used for this study. One hundred and seven samples were already genotyped for a previous study (Chapter 2), though the previous study made no attempt to analyse population structure within the South African sample. Thirty-five new samples (by-catches 1999-2000) were analysed for the first time in this study. Samples were from two different sources:

a) Samples from the shark nets. Eighty-six skin samples were collected from animals captured incidentally between 1994 and 2000 in shark nets along the KZN coast, South Africa (Fig.1). To test the geographic versus the temporal population structure hypotheses, based on the data and the information available regarding the population dynamic in the area, these samples were further subdivided into two main groups:

- samples from the North coast (from Richard Bay to Ifafa, 39 samples) representative of the resident population.

- samples from the South coast (south of Ifafa, 47 samples) expected to be from both the resident and the migratory population at least during the 'sardine run period' (SRP). Therefore, these samples were further subdivided into: -samples caught outside the SRP (representative of the putative resident population, 12 samples); -samples caught during the SRP (mixed, 35 samples).

b) Biopsy samples: 56 biopsy dart samples were taken from bottlenose dolphins moving north in large groups in excess of 500 dolphins along the Wild Coast (20km south or more of the KZN-Eastern Cape border). Samples were taken during the period June-October 1995 (during the SRP). Based on the area of sampling, the characteristics of the groups and the period of sampling, these samples were considered to be from the migratory stock.

Morphological analysis

Data regarding the degree of ventral spotting were available for 22 individuals caught in the shark nets (11 from the North Coast and 11 from the South Coast).

Ventral spotting was classified as heavy, medium, light and absent. Samples were divided in two classes: heavy-medium (3 form the North Coast and 5 from the South Coast); light-absent (8 from the North Coast and 6 from the South Coast). Multidimensional scaling analysis based on pairwise genetic distances among individuals and F_{ST} (see below) were used to test any differentiation between these two classes.

Microsatellite and mtDNA analyses

Samples were stored in salt saturated 20%DMSO. DNA was extracted by a standard phenol/chloroform extraction method (Hoelzel, 1998).

Samples were genotyped at 9 microsatellite loci: KWM1b, KWM2a, KWM2b, KWM9b, KWM12a derived from *Orcinus orca* (Hoelzel *et al.*, 1998), EV37Mn from *Megaptera novaeangliae* (Valsecchi & Amos 1996), TexVet5, TexVet7 and D08 from *Tursiops truncatus* (Rooney *et al.*, 1999, Shinohara *et al.*, 1997). PCR conditions were as reported in Chapter 2. Amplified DNA was analysed for length variation on 6% polyacrylammide denaturing gels using fluorescent imagining on an automated ABI PRISM 377 DNA sequencer, after incorporation of 1/10 concentration fluorescent labelled primer An internal standard marker (Genescan-500 ROX, Applied Biosystems) was used to determine the allele sizes.

The mitochondrial DNA control region was amplified with universal primers MTCRf (5'-TTC CCC GGT GTA AAC C) and MTCRr (5'-ATT TTC AGT GTC TTG CTT T) after Hoelzel (1998). Twenty-seven samples were sequenced for the first 599 bps at the 5' end of the mtDNA control region. Thirty-three samples were already sequenced for the same region (Chapter 2). In total 50 sequences were used. The PCR reaction conditions and PCR cycling profile were as reported in Chapter 2. PCR products were purified with QIAgen PCR purification columns and sequenced directly using the ABI dye-terminator method. Sequence alignment was performed using SEQUENCHER 3.0 (Gene Code Corp.).

Data Analysis

For the microsatellite data, observed (H_o) and expected (H_e) heterozygosities were calculated using the program ARLEQUIN 2.0 (Schneider *et al.*, 1999). Allelic richness was calculated using the program FSTAT 2.9.3 (Goudet, 2001). Deviation from the Hardy Weinberg equilibrium was tested using Fisher's exact test and the

Markov chain method (dememorization number, number of batches, iteration per batch set at 1,000, Bonferroni correction applied).

Population differentiation both for microsatellite and mtDNA data was estimated as F_{ST} (Weir & Cockerham, 1984; Michalakis & Excoffier, 1996) using the program ARLEQUIN 2.0 (Schneider *et al.*, 1999). Gene and nucleotide diversities for the mtDNA data were estimated using the same programme.

The most probable number of putative populations (K) that best explains the pattern of genetic variability was estimated using the program STRUCTURE 1.0 (Pritchard *et al.*, 2000). We assumed the admixture model and performed the analysis considering the correlated allele frequency model (burn in length and length of simulation set at 1,000,000 repetitions). To test the convergence of the priors and the appropriateness of the chosen burn in length and simulation length, we ran a series of independent runs for each value of K (for 1<K>4) as suggested by Pritchard *et al.*, (2000).

An assignment test based on the Bayesian method was performed using the programme GENECLASS 1.0.02 (Cornuet *et al.*, 1999) available at the site http://www.ensam.inra.fr/CBGP. Settings: number of simulated individuals per population 10000, rejected if probability p<0.001.

Multidimensional scaling analysis was performed using the programme *XL*-Stat Pro 6.0. The analysis was conducted using two different genetic distances: the coefficient of kinship (Cavalli-Sforza and Bodmer, 1971) and the proportion of shared alleles (Bowcock *et al.*, 1994), and analysed for 6 dimensions. The pairwise individual matrixes were calculated using the programme MSA (MicroSatellite Analyser) (Dieringer & Schlötterer, 2002). For comparative purposes only, a line was drawn through the center of the MDS distributions, bisecting each to maximize the apparent difference between the two halves.

Spatial autocorrelation analysis was performed using the programme SGS (Degen *et al.*, 2001). The geographic coordinates of the shark nets were used and a correlogram using the Moran's index was produced. Different analyses were run using different number of classes of distance (from 3 to 10). The analyses were performed setting 500 permutations and considering a confidence interval of 95%.

A median-joining network was generated to infer phylogenetic relationships among the mtDNA haplotypes using the program Network 2.0 (Bandelt et al., 1999; www.fluxus-engineering.com).

Chapter 4

Results

Microsatellite genetic diversity

A relatively low level of polymorphism (compared to other populations, see Chapter 2) was detected across the nine microsatellite loci analysed (Table 1).

Table 1. Summary of microsatellite data: number of alleles detected in each group of samples and for each locus. Allelic richness (All Rich), observed (Ho) and expected (He) heterozygosities for each population for each locus and the average are also reported. Significance levels were applied using the Bonferroni correction. *, significant at the 5% level.

Alleles	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	All Rich	Ho	He
KWM1b																		
NorthCoast	1	1	46	26												3.84	0.46	0.52
SouthCoast	0	2	42	50												2.93	0.60	0.52
Biopsies	0	1	65	46												2.61	0.46	0.52
KWM2a																		
NorthCoast	21	47	3	3												4.00	0.38	0.54
SouthCoast	12	74	2	6												3.93	0.28	0.38
Biopsies	21	82	1	8												3.61	0.39	0.44
KWM2b																		
NorthCoast	2	70	4													2.99	0.11	0.15
SouthCoast	0	83	11													2.00	0.23	0.23
Biopsies	0	100	12													2.00	0.21	0.21
KWM9b																		
NorthCoast	12	0	0	31	18	10										4.00	0.71	0.71
SouthCoast	9	1	0	34	41	7										4.74	0.65	0.67
Biopsies	17	0	4	47	31	13										4.98	0.71	0.72
KWM12a																		
NorthCoast	0	7	20	2	2	8	1	3	26	0	0					8.00	0.62	0.76
SouthCoast	0	10	13	5	0	5	4	6	47	3	1					8.70	0.77	0.72
Biopsies	1	22	16	4	1	15	1	9	41	2	0					8.65	0.84	0.79
EV37Mn																		
NorthCoast	0	0	9	18	21	12	0	9	1	1	0	2	0	0	0	7.94	0.74	0.81
SouthCoast	1	0	6	33	16	14	1	15	2	0	1	0	1	3	1	10.52	0.81	0.80
Biopsies	0	1	16	29	29	21	1	12	0	0	1	1	0	0	0	7.47	0.71	0.80
TexVet5																		
NorthCoast	2	20	31	2	11	8										5.99	0.84	0.73
SouthCoast	0	12	51	6	10	11										5.00	0.69	0.64
Biopsies	2	26	48	4	17	12										5.82	0.69	0.71
TexVet7																		
NorthCoast*	1	1	28	42	1	3										5.68	0.58	0.57
SouthCoast	0	2	49	43	0	0										2.93	0.49	0.54
Biopsies	0	1	74	37	0	0										2.61	0.41	0.48
D08																		
NorthCoast	0	1	75	2	0											2.86	0.08	0.10
SouthCoast	1	1	87	3	0											3.46	0.11	0.13
Biopsies	3	0	107	1	1											3.16	0.09	0.09
Average ± sd																		
NorthCoast																5.03 ± 1.97	0.5± 0.27	0.52 ± 0.26
SouthCoast																4.91 ± 2.86	0.51 ± 0.25	0.51 ± 0.23
Biopsies																4.54 ± 2.34	0.5 ± 0.25	0.53 ± 0.25

The number of alleles per locus ranged from 3 to 15. Allelic richness ranged from 2 to 10.52, and the average values for each group were similar. Thirteen private alleles were detected: 5 for the North Coast, 5 for the South Coast and 3 for the biopsies. Heterozygosities were similar for all groups analysed. Deviation from the

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Hardy-Weinberg equilibrium was observed only at the locus TexVet7 for the North Coast group due to heterozygote deficiency. F_{IS} values were calculated for each group (North Coast = 0.06, South Coast = -0.019, biopsy samples = 0.035).

Morphological differentiation

No differentiation was observed for the classification based on ventral spotting. The multidimensional analysis based on both the kinship coefficient and the proportion of shared alleles did not show any clustering between the two categories analysed (heavy-medium/light-absent; data not shown). F_{ST} was calculated between these categories and did not show any significant genetic differentiation (-0.003, p>0.5).

Population differentiation: resident versus migratory

The multidimensional scaling analysis showed a relatively strong difference in the proportion of North Coast and the biopsy samples either side of an arbitrary line supporting the visual pattern indicating distinct clusters (Fig. 2).

Figure 2. Scatter plot of multidimensional scaling analysis of genetic distances (proportion of shared alleles) between individuals from North Coast (◆), from the South Coast (▲) and from the biopsies (□). Dimension 1 represents 6.1% and dimension 2, 3.6% of the total variance.

This was also true for the comparison of the North Coast and the South Coast samples. In contrast, the South coast samples compared to the biopsy samples showed little evidence for assorting in different clusters. Given the arbitrary

placement of the line, these comparisons say nothing about the significance of differentiation, but serve to support the observed pattern of clustering.

The estimation of the number of populations (K) that best explain the observed genetic variability did not show any significant clustering (most probable number of populations was K=1).

F_{ST} analyses showed low but significant genetic differentiation between all pairwise comparisons of putative populations, including the comparison between the biopsy samples (putative migratory group) and the mixed South Coast group (Table 2).

Table 2. Pairwise F_{ST} values calculate with microsatellite data (matrix below) and mtDNA data (matrix above). Significance level of the p-values is reported: ns, not significant; *, significant at the 5% level, **, significant at the 1% level; *** significant at the 0.1% level.

	N	North Coast	South Coast	Biopsies
		18	15	17
North Coast	39		0.037 ns	-0.024 ns
South Coast	47	0.022***		-0.001 ns
Biopsies	56	0.009*	0.012**	

Structure of the coastal resident population

The MDS analysis showed no clear differentiation between the South Coast and the biopsy samples (Fig.2). However, any difference may be obscured by the fact that the South Coast sample will include a mixture of putative coastal and migratory populations. In order to test for the presence of a South Coast resident population differentiated from the migratory population, we performed a multidimensional scaling analysis considering only the South Coast samples. No detectable pattern was found between the individuals caught during the SRP and those caught outside the SRP. Subsequent comparison of these two groups with the biopsy group did not show any detectable pattern (in both cases dimensions 1 and 2 represented similar proportions of the total variability: 5.9% and 3.5%; 5.6% and 3.2%). This provided no support for differentiation between the migratory population and the putative resident population inhabiting the South Coast on the basis of this type of analysis.

F_{ST} values were then calculated dividing the South Coast samples into: a) samples caught outside the SRP (presumed to be from the resident population; N=12), and b) the samples caught during the SRP (presumed to be from both the

resident and migratory populations; N=35). The South Coast samples caught outside the SRP did not show significant differentiation when compared with any of the other groups tested, although small sample size will affect the reliability of these results. On the other hand, significant differentiation was observed between the South Coast samples caught during the SRP and the North Coast population (F_{ST}=0.025, p<0.01), and the biopsy samples (F_{ST} =0.012, p<0.01).

To test whether the putative South Coast resident population was the same as that inhabiting the North Coast, an assignment test was performed as an independent test using the North Coast and the biopsy groups as reference populations. The hypothesis tested was: if there was one single resident North-South coastal population differentiated from the migratory population, the individuals caught in the nets outside the SRP along the South Coast should be assigned to the North Coast group. Out of 12 individuals entrapped in the nets along the South coast outside the SRP, only 6 were assigned to the North Coast group, suggesting some level of differentiation.

The multidimensional scaling analysis supported a hypothesis of divergence between the North and the South Coast samples, showing signs of separate clustering between the North Coast and the South Coast samples caught outside the SRP similar to that described above (Fig. 3).
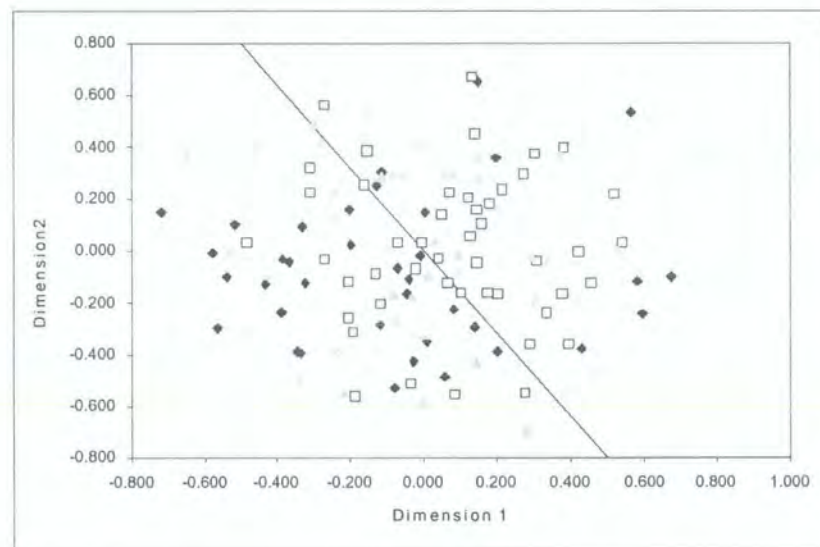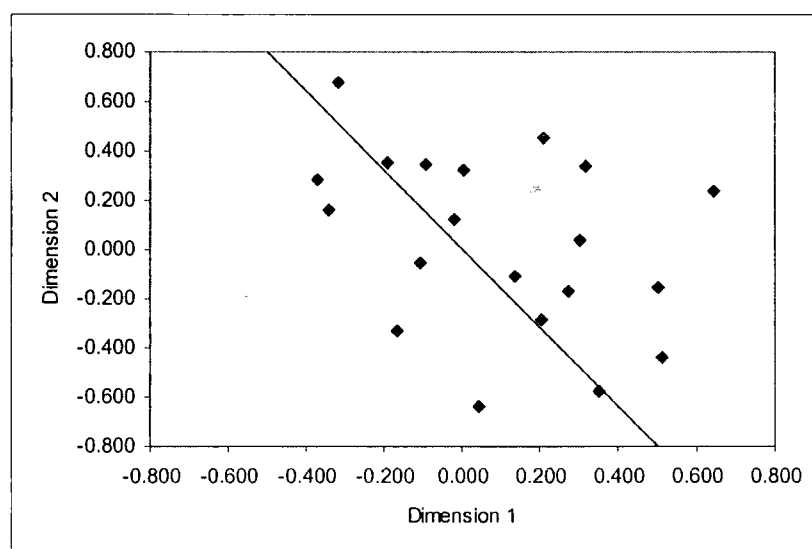

Figure 3. Scatter plot of multidimensional scaling analysis of genetic distances (proportion of shared alleles) between individuals from North Coast (◆), from the South Coast (outside SRP) (⌃). Dimension 1 represents 5.8% and dimension 2, 2.9% of the total variance.

In an attempt to identify which of the samples from the mixed South Coast sample were most likely to be from a coastal resident population we focussed on the South Coast samples caught during the SRP. An assignment test was performed to identify which individuals from the South Coast samples caught during the SRP, were from the putative South Coast resident population, and which were from the migratory population. The South Coast samples caught outside the SRP and the biopsy samples were used as reference groups. Sixteen samples out of 35 were classified belonging to the resident group, while 19 were assigned to the biopsy group. We defined a 'New' South Coast group of samples (12 plus 16 individuals) representing the putative South Coast resident population to better assess the pattern of variation along the coast. The 'New' South Coast group showed significant F_{ST} values when compared with the North Coast group ($F_{ST} = 0.027$, p<0.001) but no significant differentiation when compared with the biopsy samples ($F_{ST} = 0.003$, p>0.05).

The multidimensional scaling analysis performed on all of the samples from the nets (omitting the biopsy samples) and labelling the South Coast samples as migratory or resident according to the results of the assignment test (Fig.4) was consistent with this result.
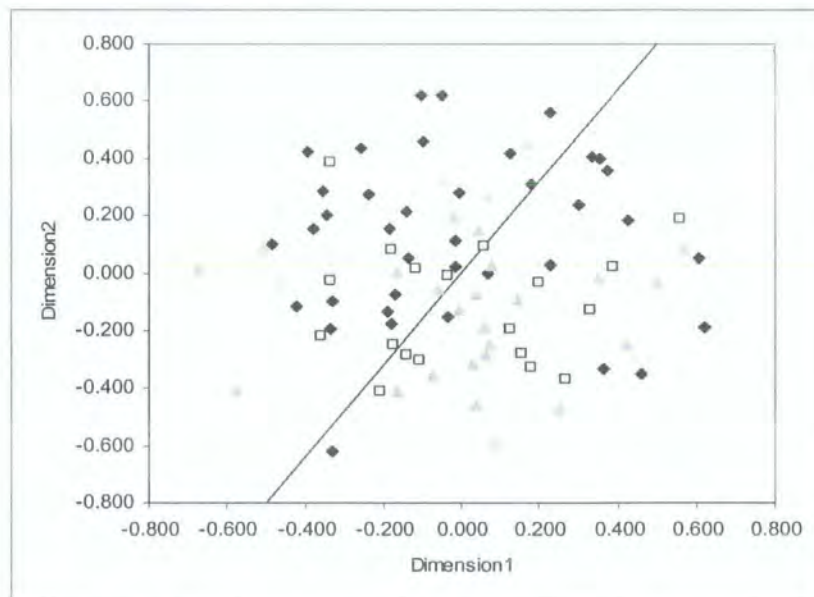
Figure 4. Scatter plot of multidimensional scaling analysis of genetic distances (proportion of shared alleles) between individuals from North Coast (◆), from the South Coast caught out of the SRP plus assigned individuals to this group (▲) and individuals assigned to the biopsy group (□). Dimension 1 represents 5.9% and dimension 2, 3.6% of the total variance.

Together these results suggested differentiation between the North Coast and the South Coast resident populations, and some differentiation between a South Coast resident population and the migratory population, though the latter is much harder to assess.

Population differentiation and geographic distance

The spatial pattern of genetic differentiation observed along the KZN coast was analysed considering the North Coast and the 'New' South Coast samples. The latter was chosen to provide a conservative representation of a putative 'coastal resident' South Coast population. All the distance classes tested showed the same trend and the best correlogram is reported in Fig.5a. The expected value for no autocorrelation was -0.012. Positive correlation between the northernmost (Richards Bay) and the southernmost regions was observed, whereas negative correlation with the North Coast was observed. The same analysis was run excluding the samples from the northernmost station (N = 2) and the positive autocorrelation was observed for distances shorter than 108 Km (Fig.5b).

a)

Correlogram using Moran's Index

b)

Correlogram using Moran's Index
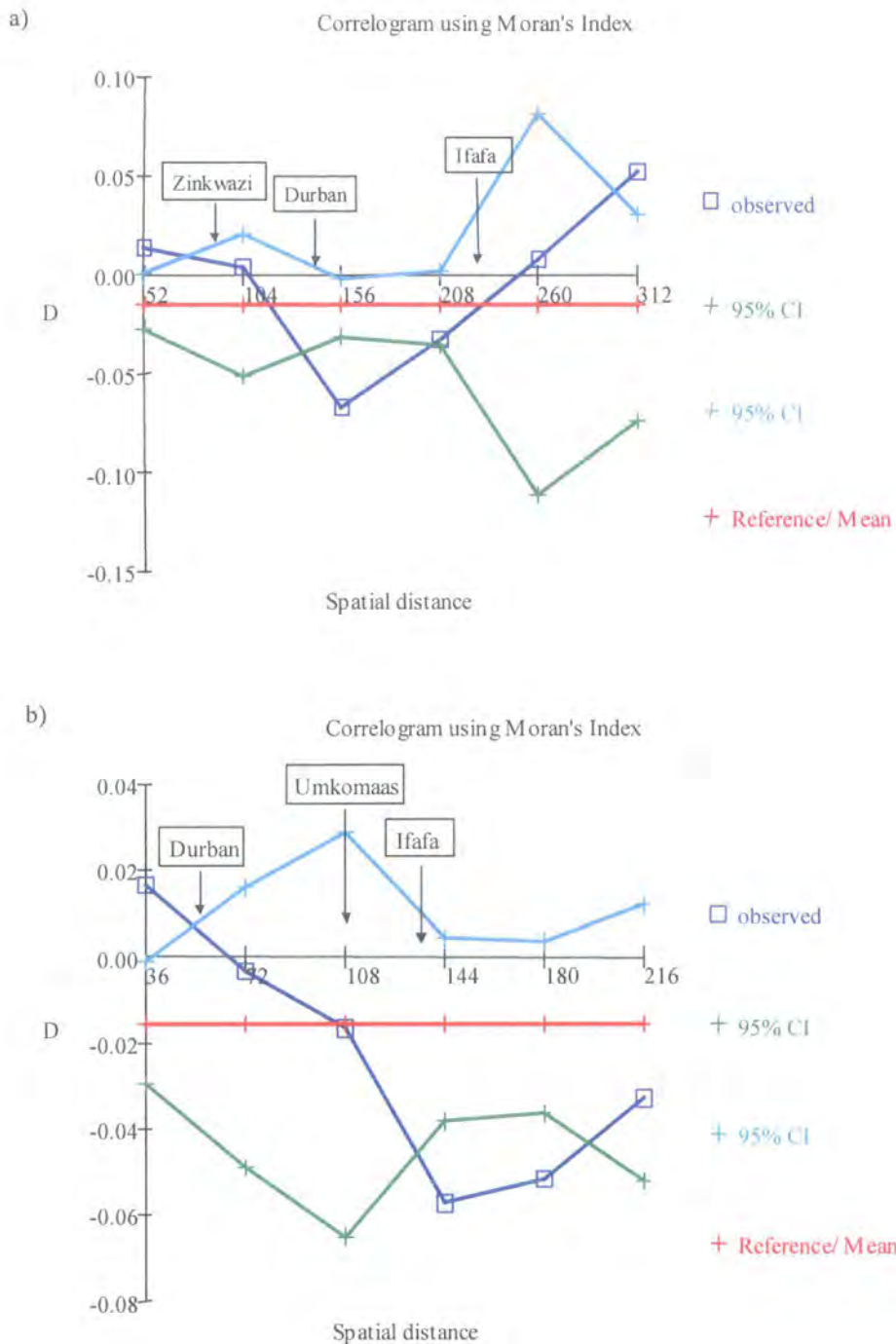
Figure 5. a) Correlogram based on Moran's index (D) calculated on the geographic distance from Richard Bay (0 Km) to Port Edward. The grey lines indicate the confidence intervals at 95%. The line parallel to the abscissa indicates the reference level of absence of spatial correlation. b) the same correlogram is calculated excluding the northernmost station of Richard Bay.

Mitochondrial DNA analysis

Mitochondrial DNA analysis was conducted on a total of 50 samples from the three groups (Table 3).

Table 3. Polymorphic sites observed among 6 haplotypes. Dots indicate identity with the reference sequence. Haplotype frequencies (right) were reported for each haplotype in each group of samples. The total number of sequences analysed for each group is reported at the bottom of the table. The asterisks indicate the central conserved domain of the control region.

Haplotypes	Polymorphic loci													Populations		
				*	*	*	*	*	*	*						
	3	3	3	3	3	4	4	4	4	5	5	5				
	5	1	1	2	3	4	5	5	9	4	4	6				
	6	0	8	6	2	7	3	4	5	7	5	8	9	North	South	Bio
Haplo1	T	A	C	T	T	G	T	G	G	T	A	T	T	10	7	10
Haplo2	C	.	.	.	.	.	.	.	.	.	.	.	.	3	7	4
Haplo3	.	.	T	.	.	A	.	A	.	C	G	C	C	4	1	1
Haplo4	.	.	T	.	C	A	.	A	.	C	G	C	C	0	0	1
Haplo5	.	.	.	.	.	.	.	.	A	.	.	.	.	0	0	1
Haplo6	.	G	.	C	.	.	C	A	.	.	.	.	.	1	0	0
Tot Haplotypes														18	15	17

Thirteen sites (2.2%) were polymorphic, identifying 6 haplotypes. All the polymorphic sites were transition substitutions. Most of the variability was observed among three haplotypes: haplotype 1, 3 and 6, while the others differ from these only for 1 base. Seven polymorphic sites (54%) were observed in the conservative region of the mitochondrial D-loop and they explained most of the difference between haplotypes 3 and 4 and the others haplotypes. The overall gene diversity was 0.627 while the nucleotide diversity was 0.0039. A median-joining network shows the phylogenetic relationship among the different haplotypes (Fig.6).
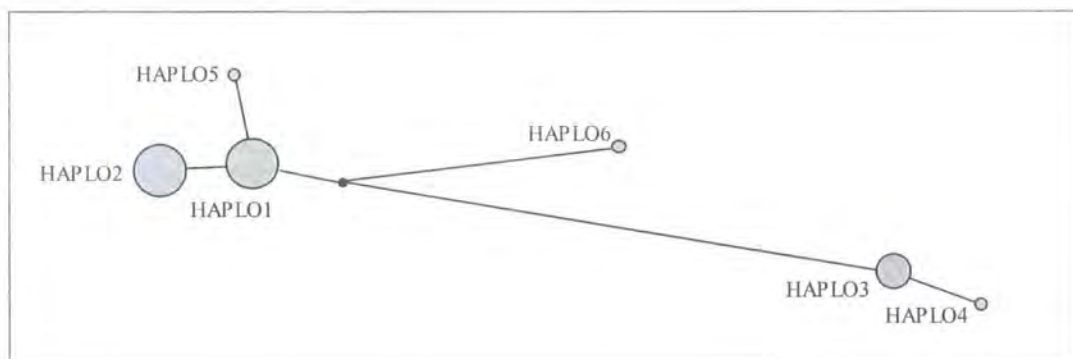
Figure 6. Median-joining network among haplotypes. Names of the haplotypes are the same as reported in Table 3. Black circles indicate ancestral extinct haplotypes.

Differentiation among the different groups considered for the microsatellite analysis was tested using the mtDNA data. Despite the occurrence of unique haplotypes in the North Coast and biopsies groups (Table 3), there were no significant F_{ST} differences between putative populations (Table 2). However, the

overall F_{ST} was also low (0.003, p>0.05, microsatellite overall F_{ST}=0.014, p<0.01), as was the nucleotide diversity, which means there would be little power for detecting differences using mtDNA for these populations. Tajima's D and Fu's Fs were respectively –0.56 (p=0.31) and 1.76 (p=0.8).

Discussion

Assessing population structure

Observational data reported two populations, resident and migratory, converging in the KZN coastal area at least for a limited period of the year (during the SRP). These populations were defined based on their different behavioural pattern: the coastal population is present along the coasts all year round, characterised by small groups, the migratory population occurs during the SRP and is characterised by larger groups pursuing the migrating fish stocks. The genetic data suggested a more complex pattern where there may be some differentiation between migratory and coastal stocks, but the clearer pattern seemed to be differentiation between northern and southern stocks along the coastal range.

Variation in ventral spotting has been observed, especially in the animals from the shark net bycatch along the southern coast. We used a small sample of extreme morphotypes (heavy-medium vs. light-absent spotted) in a preliminary attempt to test an association between genetic and morphological differences, and found none. This may be due to the small sample available.

Our result identified a North Coast population genetically differentiated from the South Coast and migratory populations. The F_{ST} and the multidimensional scaling analysis were consistent, both indicating small but significant differentiation for the North Coast samples (Table 2 and Fig.2).

From the observational data, during the SRP the migratory population moves only into the South Coast area of KZN (not further north than Ifafa). Therefore, we could not necessarily consider the South Coast samples as representative of one single population but possibly consisting of individuals from both a putative coastal and migratory populations. Based on this assumption, the South Coast samples caught outside the SRP were considered as representative of the putative South Coast population, and the South Coast samples caught during the SRP were considered mixed.

The multidimensional scaling analysis (Fig.3) and the assignment test (see results) indicated differentiation between the North Coast population and the South Coast samples caught outside the SRP (i.e. the South Coast population). However, the F_{ST} pairwise comparisons showed no significant differentiation between the South Coast samples caught outside the SRP and any of the other groups. However, the small sample size of this group (12 individuals) is likely to have affected the statistical reliability of this result.

The assignment test performed on the South Coast samples caught during the SRP allowed us to assess which individuals had higher probability of being from a South Coast population (identified by the South Coast individuals caught outside the SRP) as opposed to the migratory population. We then redefined the South Coast population (New South Coast) and the migratory population and we tested their genetic differentiation. Both the multidimensional scaling analysis and the F_{ST} analysis reconfirmed the genetic differentiation between South Coast and North Coast, but did not support genetic differentiation between the South Coast and the migratory population (Fig.4). Even so, F_{ST} comparisons between either the North Coast or the full South Coast (including samples both during and outside the SRP) and the biopsy samples (representing the putative migratory population) showed significant differentiation. Given our sample, we cannot therefore rule out differentiation between a migratory and coastal stock in the south, but our stronger data is for differentiation along the coast between the north and south.

To further quantify this, and to assess where a boundary may lie, we used spatial autocorrelation analysis. A positive correlation was found within the North Coast, while a negative correlation was found between northernmost station (Richards Bay) and both the North Coast and the South Coast. Although represented by just two samples, the Richards Bay genotypes were quite different. The suggestion is that there may be further subdivision of coastal populations further north, but an assessment of this would require further samples and analyses. Richards Bay shows different ecological characteristics compared to the rest of the coast mainly due to the presence of an estuary at the Tugela River (one of the biggest rivers of the area). The presence of bottlenose dolphins in the Tugela River estuary is lower in comparison to the rest of the North Coast, and further research is merited to determine if this may represent a population boundary for dolphins in this region, as has been suggested (Cockcroft, 1990).

The spatial autocorrelation analysis that omitted the two Richards Bay samples from the analysis suggested Umkomaas as a possible division point between the North and South Coast populations (Fig 5b). Umkomaas is about 15 Km northern Ifafa and it lies on the estuary of the Umkomaas River. There is also a significant reef system at Umkomaas called the Aliwal Shoal. Although this river is not amongst the biggest rivers of the areas, the river together with the shoal may influence the surrounding marine environment. Our result strengthens the conclusion of previous studies based on observational data that identified Ifafa as possible border between different coastal bottlenose dolphin populations (Cockcroft *et al.*, 1990).

Population history

The overall variability observed for the mtDNA locus was very low: in the Chinese *aduncus*-type population Wang *et al.* (1999) reported gene and nucleotide diversities of 0.93 and 0.016 respectively, whereas in our population these values are 0.627 and 0.0039. The median-joining network identified four primary haplotypes highly divergent form one another (Fig.6), and two other haplotypes that differ from the main types by just one base pair (Table 3). This pattern suggests a stochastic event that depleted the original haplotype diversity, leaving several remnant haplotypes and some diversity acquired since then. The distribution of the variable sites also reinforces this hypothesis, as most of the nucleotide variation is found in the central 'conserved' domain of the control region (Table 3). While the phylogenetic pattern is consistent with this founder – expansion scenario, there were no significant results from tests for possible expansion using tests for neutrality (Tajima's D and Fu's Fs). However, a relatively low level of variation at microsatellite DNA loci (Chapter 2) is consistent with a founder scenario.

If a founder model is correct, there may have been little time for differentiation among local populations. In this case low levels of divergence would reflect a short duration of separation as opposed to high levels of gene flow. There is some indication that the putative populations described here are separated by habitat boundaries, which would be consistent with the stronger evidence of divergence seen for populations over a similar geographic range in the Mediterranean Sea (Chapter 3). The populations in the Mediterranean may be older.

Conservation implications

In KZN it has been demonstrated that the presence of shark nets is having a strong impact on the depletion of this species, due to the high number of incidental by-catches. Our study clarifies the bottlenose dolphin population structure along the KZN Coasts. Small but significant differentiation was found among all putative populations. The South Coast population shows admixture with the migratory population that occurs in the area during the SRP. The geographic border between the two coastal populations was suggested by the spatial autocorrelation analysis to be around Umkomaas, north of Ifafa, consistent with previous studies. Therefore, the relevance of the number of by-catches in the shark nets has to be considered in this scenario. Along the North Coast the impact of the shark net might be relevant for the local population due to its tendency to be geographical isolated. On the other hand, along the south coast the high number of catches during the SRP could have a negative impact on population growth.

In general the state of the South African bottlenose dolphin population, as a whole, should be a cause for concern. In a worldwide phylogenetic study on the bottlenose dolphin, the South African population showed high genetic isolation, to the degree that it could be considered as a different species (Chapter 2). The low genetic variability detected in this study combined with these previous results enhances the risk of further genetic depletion of this population and therefore immediate conservation measures should be taken.

Acknowledgments

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THE COMMON DOLPHIN

Delphinus delphis – short-beaked form

Delphinus capensis – long-beaked form

CHAPTER 5

Phylogeography and phylogeny of the common dolphin (*Delphinus* sp.) based on microsatellite and mtDNA analyses

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Abstract

The common dolphin is worldwide distributed and shows a high degree of morphological variation. Two distinct morphotypes, long-beaked and short-beaked, have been considered different species named *D. capensis* and *D. delphis*, respectively. However, genetic differentiation between these two forms has only been proved in the Pacific. We analysed samples from eight different areas, including two morphologically defined long-beaked form populations, and we compared them with the eastern North Pacific populations. Both mitochondrial DNA and microsatellite markers showed the same pattern of differentiation suggesting similar movements of females and males. We found high differentiation among the populations described as long-beaked and no monophyly suggesting that these populations may have evolved from independent events converging to the same morphotype. We observed low genetic differentiation among the short-beaked populations, suggesting that gene flow even among distant geographic areas is considerable or in some cases that population divergence is recent.

Keywords: common dolphin, population genetic, microsatellites, mtDNA, molecular ecology, phylogeography

Introduction

The common dolphin is widely distributed in all oceans, from temperate to tropical waters and shows high mobility across its habitat. It shows such a high degree of geographic variation in morphology that more that 20 different species were described in the past, although they have all subsequently been considered local variations of one single species *Delphinus delphis* (Hershkovitz, 1966). The present classification within this genus is still uncertain, although two different species are generally accepted: a long-beaked form (*Delphinus capensis*) and a short-beaked form (*Delphinus delphis*).

The separation between these two forms is based on both external morphological characters, such as the colour pattern and overall body size, and skeletal morphological characters, including the length of the rostrum, the tooth and the vertebra counts (Heyning & Perrin, 1994). Specific status for these two forms was based largely on the morphological and genetic analysis of two sympatric populations occurring along the coast of California. In this area, the bigger long-beaked form prefers coastal shallow waters, whereas the smaller short-beaked form occurs mainly in deeper oceanic waters. From the analysis of the mtDNA control region sequences, Rosel *et al.* (1994) found reciprocal monophyly, genetic divergence of 1.11 %, and fixed differences. Comparison between short-beaked individuals from southern California and short-beaked individuals from other oceans (eastern tropical Pacific and Black sea) differed by only 0.02 % (Rosel *et al.* 1994).

A similar pattern has been observed in other species. Recently, in the genus *Stenella* different species have also been recognised based on morphological differences (*Stenella clymene* versus *Stenella longirostris* (Perrin *et al.*, 1981) and morphological differences have been reported between offshore and nearshore populations of spotted dolphins (*Stenella attenuata*) (Douglas *et al.*, 1984).

The bottlenose dolphin (genus *Tursiops*) shows a high degree of morphological variation and despite its capacity for long distance dispersion and its flexible social structure, strong differentiation has been reported even between sympatric populations (Mead & Potter, 1995, Hoelzel *et al.*, 1998). Different species have recently been recognised within this genus: the coastal *aduncus* form in Chinese waters (Wang *et al.*, 1999) and the South African *aduncus* form (Chapter 2). Some authors (Hoelzel *et al.* 1998, Chapter 2) suggest that specialisation for local resources by the consolidation of different feeding strategies among groups might favours the

residency of the individuals belonging to a population, and lead to the progressive divergence of the groups.

However, for the common dolphin the classification *delphis/capensis* remains controversial. Exceptions to this subdivision have frequently been found in several areas. In southern Australia a recent study showed the existence of two ecotypes inhabiting shallower and deeper waters. However, morphological data suggest one single population that exhibits high morphological variability: the rostral length-zygomatic width ratio was found spanning the range of both species found in the eastern North Pacific. Conversely to the situation observed in the eastern North Pacific, the bigger form was observed mainly in deeper colder waters whereas the smaller in warmer coastal waters (Bell *et al.*, 2002). Preliminary genetic analysis suggested no differentiation between the two ecotypes and classified both as *Delphinus delphis* (White, 1999).

High variability of the rostral length-zygomatic width ratio is also observed in the eastern North Atlantic where the species is known to be the short-beaked form. Recent morphological analysis found that the rostral length-zygomatic width ratio, the condylobasal length, and the body size overlapped the range of both the short and long-beaked forms described in the eastern North Pacific. However, based on coloration patterns, tooth count, and length of the rostrum, this population was described as a large-form of the short-beaked form (Murphy, 2004).

Morphological differences have also been reported within the long-beaked form. The South African long-beaked, although very similar to the Pacific long-beaked form in coloration, rostral length, and tooth count, differs for the average total vertebra count (Heying & Perrin, 1994). In the Indo-Pacific (form Middle East to China) an extremely long-beaked form has been observed with evidence of clinal variation in the size as one moves east or west from India. This form has been named *Delphinus delphis tropicalis* Van Bree 1971 and suggested as possible subspecies of the *capensis* (Jefferson & Van Waerebeek, 2002). Possible paraphyly of the Indian and Pacific long-beaked form has also been suggested by Le Duc *et al.* (1999) and reinforced by Jefferson & Waerebeek (2003) that suggested differentiation between the eastern Pacific long beaked common dolphin and the Indo-Pacific *D. capensis*.

Data in literature suggests different geographic distribution for the two forms and few areas where they overlap their range (Perrin, 2002). The short-beaked common dolphin shows a continuous distribution north-south along both the eastern

and the western coasts of the Atlantic Ocean, and the eastern coast of the Pacific Ocean. It is also present from central Japan to Taiwan, around New Caledonia, New Zealand and Tasmania in the western Pacific, and in the Mediterranean and Black Seas. Conversely the long-beaked form occurs disjunctly along the western coast of Africa, South Africa and Madagascar, along the eastern coast of South America (from Venezuela to Argentina), from southern California to central Mexico in the eastern Pacific, around Korea, southern Japan and Taiwan in the western Pacific. The form *D. tropicalis* is observed only in the northern Indian Ocean and Southern Asia (Perrin, 2002). In general the short-beaked form occurs mainly offshore, although coastal resident populations have been described in the Mediterranean Sea (Greece) and eastern North Atlantic.

Little is known about the social structure of either of these species. *Delphinus delphis* is generally identified as a pelagic species living in groups of 500-1000 individuals. It has been suggested that large groups might be structured in smaller subgroups of 20-30 individuals, possibly including kin (although there is no evidence to support this; Evans, 1994) and that subgroups might be based on age or sex (Perrin, 2001). A photoidentification study on a small coastal population of short-beaked common dolphins inhabiting the waters around Kalamos (Greece) suggested a fission-fusion type of society, similar to the structure found in the bottlenose dolphin (Bruno, 2001).

In this work we analysed populations from eight different regions across the Atlantic and Indian oceans and compared these with the published data of the two populations of the eastern North Pacific Ocean. We test whether the long-beaked form can be considered a single species worldwide. One possibility is that the long beaked form originated from one single event in one area and subsequently spread in different regions. In this case we expect to find monophyly among the long-beaked populations compared to the short-beaked populations and strong differentiation between long-beaked and short-beaked populations. A second possibility is that the long-beaked form originated independently in different regions. The selection of this morphotype may be the result of adaptation to local environments. Moreover, we address the question of how population structure may have evolved in a highly mobile marine species, given the pattern of differentiation observed. Considering the behavioural ecology of the common dolphin we expect low population differentiation even on a world wide scale.

Materials & methods

Sample collection and DNA extraction

In total, 200 *Delphinus* sp. samples from 8 geographic regions were analysed in this study (Fig. 1).

Samples from the eastern North Atlantic (Galicia, Celtic Sea, and Scotland), the eastern central Atlantic (Açores, Canary Islands and Madeira) and western North Atlantic were described as short-beaked form (*Delphinus delphis*). Samples from South Africa were described as long-beaked form (*Delphinus capensis*). Samples from Mauritania were described as long-beaked form, although the comparison of the skull measurements with the published data in Heying and Perrin (1994) classified the individuals as follows: MAU1, very likely short-beaked; MAU2 clearly short-beaked; MAU3 and MAU4, likely long-beaked; MAU5, clearly long-beaked, MAU6, unclear; MAU7 uncommon very short-beak (Aguilar, unpublished data). Samples from Argentina were labelled *D. delphis*, although the actual form was not determined (Table 1). Samples were obtained from stranded dolphins or dolphins accidentally caught in nets. Samples from Mauritania and two samples from the Açores were bone specimens.

Table 1. List of the populations analysed and correspondent acronyms. The number of samples for each population considered in this article are reported for the microsatellite and the mtDNA analyses. Data taken from other publications are as follows: a) from Chapter 6, b) four sequences from Açores were from Matzen Silva *et al.* (submitted), c) from Rosel *et al.* (1995).

Population	Acronym	Microsatellite	mtDNA
Galicia, Gulf of Biscay	**GAL**	39[a]	36[a]
Celtic	**CEL**	41	29
Scotland	**SCO**	26	21
Eastern Central Atlantic	**ECA**	13	14[b]
Western North Atlantic	**WNA**	13	11
Mauritania	**MAU**	-	7
Argentina	**ARG**	18	15
Short-beaked Pacific	**sbPA**	-	13[c]
Long-beaked Pacific	**lbPA**	-	11[c]
Long-beaked South Africa	**lbSA**	43	20
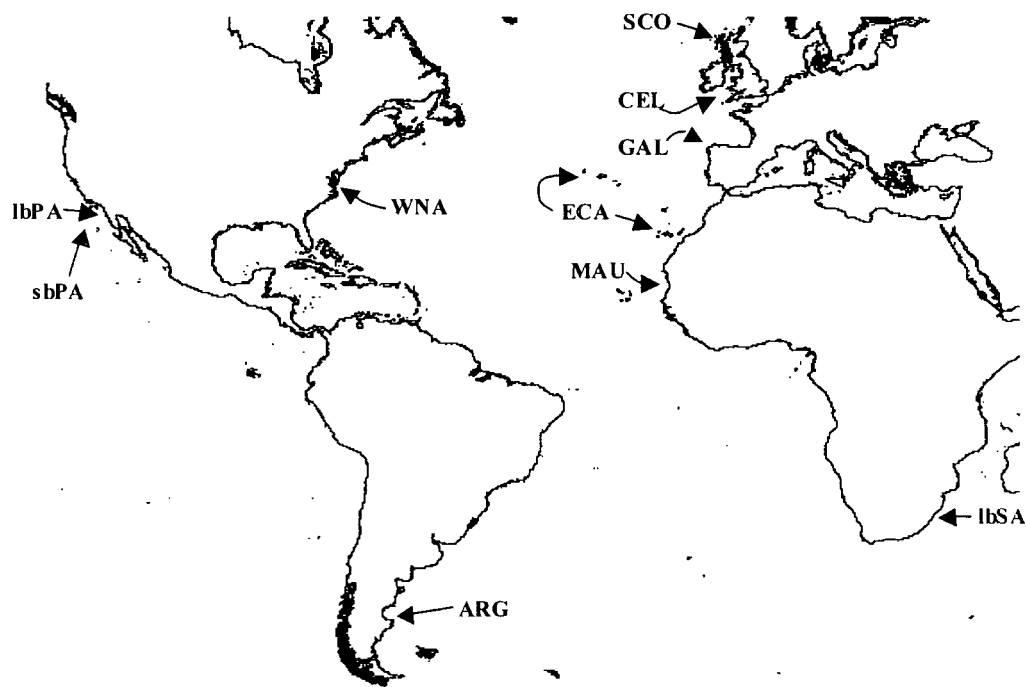
Figure 1. Map of sample locations. Abbreviations are as in Table 1.

DNA was extracted from tissue samples preserved in salt saturated 20% DMSO by a standard phenol/chloroform extraction method (Hoelzel, 1998). DNA was extracted from bone samples using QIAgen PCR purification columns after grinding 100mg of bone and digesting it at 37°C for 48 hours in 1ml of digestion buffer (0.01M TRIS, 0.01M NaCl, 1% SDS, 2mg/ml proteinase K, 0.01 PTB). The extraction and the analysis of the bone specimens were conducted in a different laboratory where no cetacean DNA had ever been manipulated before, to avoid contamination. An extraction including everything but tissue was carried through all the analyses as negative control.

Sex determination

Individuals whose gender was unknown were sexed amplifying portions of the genes ZFX and ZFY as described in Bérubé and Palsbøll (1996).

Microsatellite analysis

Nine published microsatellite loci were analysed. Primers KWM1b, KWM2a, KWM2b, KWM9b, KWM12a were derived from *Orcinus orca* (Hoelzel *et al.*, 1998), EV37Mn from *Megaptera novaeangliae* (Valsecchi & Amos 1996), TexVet5, TexVet7 and D08 from *Tursiops truncatus* (Rooney *et al.*, 1999, Shinohara *et al.*,

1997). Microsatellites were not successfully amplified on the Mauritania samples, due to the highly degraded status of the DNA extracted from these samples and the relatively large size of most of the microsatellite markers. Amplified DNA was analysed for length variation on 6% polyacrylamide denaturing gels using fluorescent imagining on an automated ABI PRISM 377 DNA sequencer, after incorporation of 1/10 fluorescent labelled primer (PCR reaction conditions: 100μM dNTPs, 0.75-1.5 mM MgCl$_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 200nM of each primer, 0.02 U/μL Taq polymerase. PCR cycling profile: 5 min at 95°C; then 35 cycles of 40 sec at 94°C, 1min at the T°ann, 1 min at 72°C; then 10 min at 72°C). The annealing temperatures (T°ann) were as follows: KWM1b: 48°C; KWM2a: 48°C; KWM2b: 44°C; KWM9b: 62°C; KWM12a: 56°C; EV37Mn: 52°C; TexVet5: 49°C; TexVet7: 49°C; D08: 57°C. An internal standard marker (Genescan-500 ROX, Applied Biosystems) was used to determine the allele sizes.

For microsatellite loci, the level of polymorphism was estimated as the number of alleles per locus, observed heterozygosity (H$_o$), expected heterozygosity (H$_e$), and allelic richness. Allelic richness controls for variation in sample size by a rarefaction method, and was calculated using the program FSTAT 2.9.3 (Goudet 2001). Evaluation of possible deviations from the expected Hardy-Weinberg (HW) equilibrium (overall deviation, heterozygote deficiency and heterozygote excess) were performed using Fisher's exact test and the Markov chain method (dememorization number, number of batches, iteration per batch set at 1,000, Bonferroni correction applied).

Genetic differentiation among populations was assessed based on both the infinite allele model (IAM) using F$_{ST}$, and the stepwise mutation model (SMM) using Rho$_{ST}$. The level of differentiation between population pairs was estimated as F$_{ST}$ (Weir & Cockerham, 1984) using the program ARLEQUIN 2.0 (Schneider *et al.*, 1999) and Rho$_{ST}$ using the program RstCalc (Goodman, 1997). The significance of the difference of F$_{ST}$ and Rho$_{ST}$ values from zero was tested by permutation analysis. A permutation test to assess differentiation for allele size was performed for F$_{ST}$ and Rho$_{ST}$ using the program SPAGeDi (Hardy *et al.*, 2002).

The most probable number of putative populations (K) that best explains the pattern of genetic variability was estimated using the program STRUCTURE 2.1 (Pritchard *et al.*, 2000). We assumed the admixture model and we performed the analysis considering both the independent and the correlated allele frequency model.

Burning length and length of simulation was set at 100000 and 1,000,000 repetitions, respectively. To test the convergence of the priors and the appropriateness of the chosen burn in length and simulation length, we ran a series of independent runs for each value of K (for 1<K>7) as suggested by Pritchard *et al.* (2000). We tested whether any particular individual was an immigrant or had an immigrant ancestor, by using the model with prior population information, subdividing the individuals into K populations, according to the results of the previous analysis. We assumed v (migration rate) = 0.05 and 0.1, and testing for 0<number of generations (G)>2.

Sex-biased dispersal was tested using the program FSTAT 2.9.3 (Goudet, 2001). Only adult individuals (a total of 187: 73 females and 114 males) were considered for this analysis.

An asymmetric estimate of the migration rate ($M=4N_em$) between pairwise populations, based on microsatellite and mtDNA data, was calculated using MIGRATE (Beerli, 1997-2002). The length of the runs was optimised for both markers (acceptance-rejection > 2%, R<1.2). Initial runs were set estimating θ and M with F_{ST} and allowing M to be asymmetric. Reruns were set using the parameter estimated found with the first run and lengthening the MCMC chains. In order to verify the result a final run was set using longer chains. For comparison the migration rate was also calculated by hand according to $F_{ST}=1/(4Nm+1)$.

mtDNA analysis

The first 369 bps at the 5' end of the mtDNA control region were sequenced in a total of 150 samples, while other sequences were obtained from the published databases (Table 1). In total 178 sequences of *Delphinus* sp. were available.

The mitochondrial DNA control region was amplified either with universal primers MTCRf (5'-TTC CCC GGT GTA AAC C) and MTCRr (5'-ATT TTC AGT GTC TTG CTT T) after Hoelzel (1998), or with the primers 5'-ACA CCA GTC TTG TAA ACC-3' and 5'-TAC CAA ATG TAT GAA ACC TCA G-3' after Rosel *et al.* (1994).

The PCR reaction conditions were as follows: 100μM dNTPs, 1.5 mM MgCl$_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 200nM of each primer, 0.02 U/μL Taq polymerase. The PCR cycling profile was 4 min. at 95°C, 35 cycles of 45 sec. at 94°C, 1.5 min. at 50°C, and 1.5 min. at 72°C, followed by 8 min. at 72°C. PCR

products were purified with QIAgen PCR purification columns and sequenced directly using the ABI dye-terminator method.

Mitochondrial DNA from the Mauritania samples was amplified using two sets of primers designed in order to amplify two overlapping portions of the control region of approximately 200bps each (Dmtcrf: 5'-TTA GTC TCT CCT TGT AAA T-3' and Dmtcrr: 5'-GGT GAT TAA GCT CGT GAT-3'; MTCRf and mtancr: 5'-AAA ATA AAT GAA TGC ACA ATA-3'). The PCR reaction conditions were as follows: 100μM dNTPs, 2.5 mM MgCl$_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 200nM of each primer, 0.4 μg/μl BSA, 0.02 U/μL Taq polymerase. The PCR cycling profile was 15 min at 95°C, 45 cycles of 45 sec at 94°C, 1.5 min at 47°C, and 1.5 min at 72°C, followed by 8 min at 72°C.

Sequences were compared with 25 published sequences of two populations of common dolphins from the Pacific Ocean described as short-beaked and long-beaked form, respectively (Rosel *et al.*, 1994). Other four published sequences from Açores were included in the analysis (Matzen Silva *et al.*, 2002).

Sequence alignment was performed using ClustalX (Thompson *et al.*, 1997). The degree of differentiation (F_{ST} and Φ_{ST}) and Tajima's D were estimated using ARLEQUIN 2.0 (Schneider *et al.*, 1999). Estimates of Φ_{ST} used the Tamura-Nei genetic distance model (Tamura & Nei 1993).

Genetic distance (Da) was estimated using Tamura-Nei with the SENDBS programme, written by N. Takezaki (National Institute of Genetics, Mishima, Shizuoka, Japan; http://oat.bio.indiana.edu:7580/documents/public/molbio/tools/Sendbs/). SENDBS was also used to estimate π.

Individual haplotypes were compared phylogenetically by the neighbour-joining method using PAUP* 4.0b10 (Swofford, 1997) and rooted with homologous sequence from *Stenella attenuata*. Majority-rule consensus trees were constructed from 1,000 bootstrap replications and a 50% criterion for the retention of nodes was applied. Distances were based on Tamura-Nei as above. The ti/tv ratio was set at 6.5, based on observed values. A maximum parsimony phylogenetic reconstruction was based on 1,000 bootstrap replications, retaining branches with 50% support or greater. A median-joining network was generated to infer phylogenetic relationships among the mtDNA haplotypes using the program NETWORK 4001 (Bandelt *et al.*, 1999; www.fluxus-engineering.com).

Results

Microsatellite analysis

Population differentiation and diversity

Expected (He) and observed (Ho) heterozygosities were calculated for each population at each locus (Table 2).

Table 2. Number of alleles (number of private alleles in parentheses, allelic richness in square brackets), expected (He) and observed (Ho) heterozygosities for each population at each microsatellite locus. Allelic richness was calculated on a minimum number of samples of 12 individuals. One asterisk indicates those loci with a p-value < 0.05 when tested for heterozygote deficiency. Two asterisks indicate those loci still significant after Bonferroni correction (p-value <0.00079). Abbreviations are as in the caption of Table 1.

		Populations						
		GAL	**CEL**	**SCO**	**ECA**	**WNA**	**ARG**	**IbSA**
		39	41	26	13	13	18	43
KWM1b	N. Alleles 4	4 [2.6]	3 [2.66]	3 [2.18]	4 [3.92]	2 [2]	2 [1.94]	1 [1]
	Ho	0.21	0.219	0.115	0.308	0*	0.125	0
	He	0.271	0.246	0.149	0.508	0.221	0.179	0
KWM2a	N. Alleles 18	13 [10.43]	13 [8.57]	12 (1) [9.58]	11 [10.54]	8 [7.92]	11 [9.76]	9 [7.81]
	Ho	0.846	0.878	0.76	0.769	0.615*	0.889	0.93
	He	0.901	0.844	0.893	0.895	0.895	0.913	0.851
KWM2b	N. Alleles 8	7 [6.43]	6 [5.85]	6 [5.92]	6 [5.99]	6 [6]	5 [4.94]	5 [4.2]
	Ho	0.795	0.878	0.808	0.769	0.667	0.667	0.619
	He	0.825	0.824	0.827	0.84	0.837	0.756	0.61
KWM9b	N. Alleles 16	13 (1) [9.59]	11 [8.8]	9 [7.96]	10 [9.76]	10 (1) [10]	12 [10.65]	8 [6.1]
	Ho	0.897	0.927	0.769	0.923	0.917	0.937	0.805
	He	0.892	0.869	0.874	0.917	0.888	0.897	0.78
KWM12a	N. Alleles 14	9 [7.47]	10 [7.31]	9 [7.16]	8 (1) [7.77]	11 (1) [10.61]	8 [6.96]	8 [6.57]
	Ho	0.795	0.78	0.808	0.692	0.923	0.722	0.683
	He	0.791	0.805	0.771	0.831	0.905	0.825	0.815
EV37Mn	N. Alleles 31	20 [11.84]	18 [11.54]	19 (2) 12.94]	14 (1) [13.3]	12 [11.6]	11 [9.65]	12 (1) [7.11]
	Ho	0.949	0.805	0.885*	0.846	0.923	0.823	0.721
	He	0.918	0.903	0.912	0.898	0.914	0.895	0.814
TexVet5	N. Alleles 14	10 (1) [7.63]	9 [7.07]	10 [9.14]	9 [9]	8 [8]	10 (1) [8.8]	8 [6.83]
	Ho	0.553**	0.575*	0.461**	0.583*	0.667	0.667	0.762*
	He	0.846	0.803	0.903	0.931	0.851	0.88	0.84
TexVet7	N. Alleles 8	5 [4.83]	6 [4.49]	5 [4.57]	5 [4.85]	7 (1) [6.92]	7 [6.29]	4 [3.86]
	Ho	0.667	0.8	0.538	0.538	0.692	0.571	0.69
	He	0.746	0.708	0.744	0.68	0.828	0.698	0.654
D08	N. Alleles 21	16 (2) [9.74]	11 [7.45]	11 [8.61]	8 [8]	12 (1) [11.5]	13 [11.05]	8 (1) [6.04]
	Ho	0.667**	0.78	0.8	0.75	0.846	0.778	0.658
	He	0.863	0.808	0.856	0.855	0.889	0.913	0.716
Average (SD)	N. Alleles	10.78 (5.2) [7.84 (2.9)]	9.7 (4.4) [7.08 (2.6)]	9.3 (4.7) [7.56 (3.1)]	8.3 (3.1) [8.13 (2.9)]	8.4 (3.2) [8.28 (3.1)]	8.8 (3.6) [7.78 (3)]	7 (3.2) [5.5 (2.1)]
	Ho	0.71 (0.22)	0.74 (0.22)	0.66 (0.25)	0.69 (0.18)	0.69 (0.29)	0.69 (0.24)	0.65 (0.26)
	He	0.78 (0.2)	0.76 (0.2)	0.77 (0.24)	0.82 (0.14)	0.80 (0.22)	0.77 (0.23)	0.68 (0.27)

Loci that significantly deviated from the HW equilibrium were found in all populations except in the ARG population. In all cases deviation from HW equilibrium was due to significant heterozygosity deficiency (p-values<0.05). The locus TexVet5 significantly deviated from the HW equilibrium in all populations except in the ARG and the WNA populations. Omission of this locus did not change the pattern of differentiation among populations and therefore was retained in the subsequent analyses. The locus KWM1b was monomorphic in the lbSA population. The lbSA population showed the lowest average observed and expected heterozygosities and the lowest allelic diversity. The other populations showed similar values for both the average heterozygosities and allelic diversity parameters. Genetic differentiation among pairwise population was estimated using F_{ST} and Rho_{ST} (Table 3). The pattern shown by the two methods was similar, although in some cases the values for the same pairwise comparison differed. The lbSA population (long-beaked form) showed the highest differentiation compared to all the other populations, with both F_{ST} and Rho_{ST} analyses. The ARG population was also significantly differentiated from all the other populations, although Rho_{ST} values suggested no differentiation when compared to the WNA and the ECA populations. The eastern Atlantic populations (GAL, CEL, SCO, and ECA) clustered together showing low or no genetic differentiation. Rho_{ST} values showed a similar trend. The WNA population significantly differentiated from all the populations except SCO, although the Rho_{ST} analysis indicated significant values only between the WNA and CEL population and WNA and lbSA population.

Table 3. Genetic differentiation among pairwise populations using microsatellite data. F_{ST} values are reported below the diagonal, while Rho_{ST} values are reported above the diagonal. Statistical significance of the p-values is reported as follows: * for p<0.05, ** for p<0.01, ***for p<0.001.

	N	GAL	CEL	SCO	ECA	WNA	ARG	lbSA
GAL	39		0.008	0.03**	0.008	0.02	0.049**	0.164***
CEL	41	0.005		0.011	0.004	0.028*	0.088***	0.201***
SCO	26	0.012*	0.011**		0.028	0.025	0.061**	0.171***
ECA	13	-0.002	0.001	0.01		-0.019	0.032	0.12***
WNA	13	0.014*	0.023***	0.012	0.021*		-0.002	0.095***
ARG	18	0.037***	0.045***	0.034***	0.041***	0.037***		0.1***
lbSA	43	0.1***	0.105***	0.103***	0.099***	0.087***	0.076***	

We tested the role of allele size in determining population differentiation comparing the F_{ST} and Rho_{ST} values. The test suggested that allele size does not contribute to the differentiation of the populations we analysed (p-value=0.7), and therefore F_{ST} should be preferred to Rho_{ST}.

Bayesian analysis was used as alternative approach to test for population structure. The number of populations (K) that best explained the genetic variability observed across our samples was found to be three. Consistency among different runs was observed for the estimate of P(X/K) and the prior α, indicating that the burn-in length and the length of the runs were appropriate. The same result was found using both correlated allele frequency and independent allele frequency models. The Bayesian analysis identified three main clusters of populations (Fig. 2). The lbSA population highly differentiated from all the others, a western Atlantic cluster (including WNA and ARG), and an eastern Atlantic cluster (including GAL, CEL, SCO, ECA).
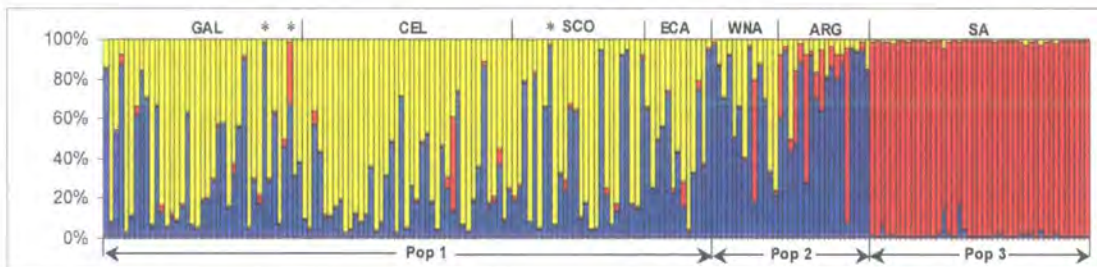
Figure 2. Estimated proportion of the coefficient of admixture of each individual's genome that originated from population *k*, for K=3 (below the graphic). Each individual is represented by a column. Geographic origin of the samples is reported above the graphic. The asterisks indicate the individuals identified as migrants.

Migrants and sex-biased dispersal

We analysed whether individuals were possible immigrants or descendants of recent immigrants considering the three populations identified by the Bayesian analysis. Three possible immigrant individuals were identified. All individuals were migrants from the second cluster (WNA, ARG) to the first cluster (GAL, CEL, SCO). Two individuals were from GAL and they were identified as possible immigrants at both the values of *v*=0.01 and *v*=0.05. One individual was from SCO and it was identified as immigrant only for *v*=0.05. All individuals had higher probabilities to be immigrants rather than having immigrant ancestry.

Although the Bayesian analysis clustered the WNA and the ARG populations in the same group, the FST values indicated significant differentiation between these two populations. Therefore, we calculated the migration rate (M) considering four main populations: the EA (eastern Atlantic) population (including GAL, CEL, SCO and ECA), the WNA population, the ARG population, and the lbSA population. The results are reported in Table 4.

Table 4. Estimate of the migration rate (M) between populations based on the microsatellite and mtDNA data. Nm columns refer to the values calculated according to $F_{ST}=1/(4Nm+1)$. The other columns refer to the asymmetrical migration rate calculated using a maximum likelihood method (MIGRATE): 1,2 stands for: migration from population 1 to population two; 2,1 stands for: migration from population 2 to population 1. The confidence interval (95% c.i.) is also reported. Abbreviations for the populations are as in Table 1 except for EA that stands for eastern Atlantic populations (GAL, CEL, SCO, ECA).

Population	microsatellites (bi-parental)				mtDNA (maternally inherited)			
	Nm	1,2	2,1	95% c.i.	Nm	1,2	2,1	95%c.i.
1 EA	17.080	--	5.210	4.83-5.62	10.040	--	20.820	9.37-35.63
2 WNA		9.360	--	8.68-10.09		1.510	--	0.26-4.0
1 EA	6.900	--	7.090	6.64-7.56	14.030	--	5.640	3.11-13.36
2 ARG		10.690	--	9.99-11.44		0.000	--	4.8e-11-0.56
1 EA	2.410	--	4.230	3.88-4.6	5.290	--	5.694	3.276-11.063
2 SA		3.900	--	3.54-4.25		0.316	--	0.24-1.09
1 WNA	6.560	--	13.400	12.46-14.41	8.020	--	0.044	0.03-0.82
2 ARG		12.940	--	12.11-13.8		9.180	--	3.34-30.29
1 WNA	2.620	--	6.190	5.48-6.88	3.620	--	0.000	1.89e-11-0.395
2 SA		2.530	--	2.26-2.82		1.600	--	0.672-3.034
1 Arg	3.030	--	6.840	6.14-7.63	5.070	--	43.897	22.698-70.852
2 SA		2.710	--	2.42-3.11		0.740	--	0.665-2.698

Sex-biased dispersal among regions was tested. No significant heterozygote deficiency or positive F_{IS} were observed for either sex (F_{IS} for females was 0.054, F_{IS} for males was 0.076, p=0.41), and the assignment index was insignificant (p=0.63, assignment index variance: p =0.52) indicating no significant sex biased dispersal among regions.

Mitochondrial DNA analysis

Population differentiation

Mitochondrial control region sequences from the seven populations analysed above were compared with sequences from Mauritania (MAU) and with published sequences from two population in the Pacific Ocean identified as long-beaked form (lbPA) and short-beaked form (sbPA).

Among the 178 sequences analysed, 96 haplotypes were identified, showing 76 polymorphic sites (Fig.3).
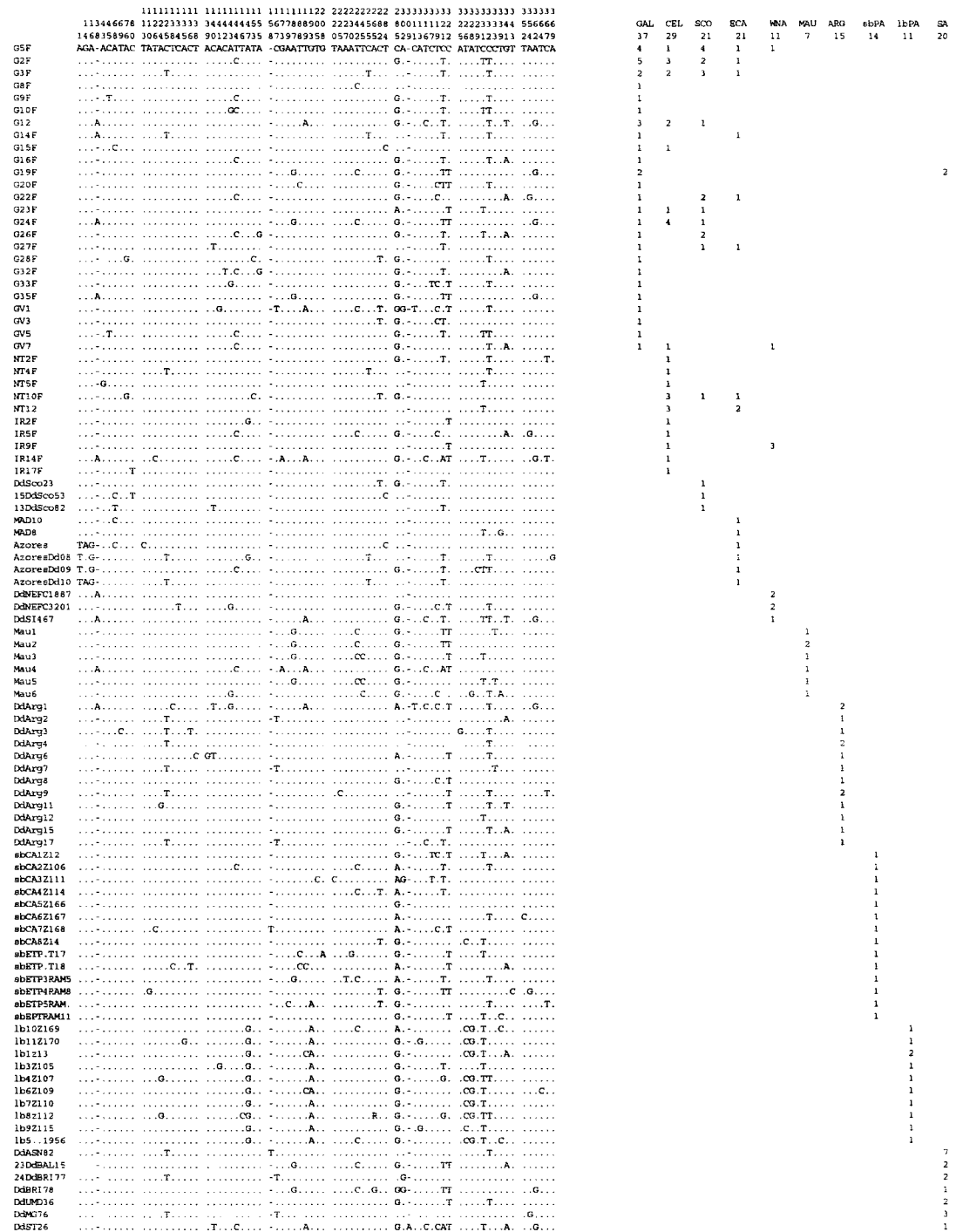
Figure 3. Polymorphic sites among 96 haplotypes (left). Dots indicate identity with the reference sequence. Haplotype frequencies (right) were reported for each haplotype in each putative population. Population abbreviations are as reported in Table 1. The total number of individuals sequenced for each population is also reported below the population name.

Shared haplotypes were common among the eastern Atlantic populations (GAL, CEL, SCO, ECA) and the WNA population. MAU, ARG and the Pacific

Ocean populations (lbPA, sbPA) did not show any shared haplotype. The lbSA population shared one haplotype with the GAL population. Five sites exhibited differences in nucleotide substitution frequency between the lbPA population and the other populations (Fig. 3).

Average gene and nucleotide diversities were estimated for each population (Table 5). Diversities were relatively high for all the populations analysed. The lbSA population showed the lowest gene diversity, whereas the lbPA population showed the lowest nucleotide diversity. Neutrality tests were performed. Although Tajima's D values were not statistically significant, Fu's Fs values were large, negative and significant for the GAL, ECA, lbPA and sbPA populations, suggesting possible population expansion (Table 5).

Table 5. Gene diversity, nucleotide diversity, Tajima's D and Fu's Fs values are reported for each population. Asterisks indicated statistically significant values (* =p-value<0.05, ** =p-value<0.01, *** =p-value<0.001).

Pop	N. seq	gene div.	nucl. div.	D	Fs
GAL	36	0.967	0.017	-0.825	-13.4***
CEL	29	0.958	0.018	-0.446	-5.6*
SCO	21	0.943	0.015	-0.697	-3.03
ECA	14	0.989	0.018	-0.533	-6.4**
WNA	11	0.909	0.013	-0.434	-0.717
MAU	7	0.952	0.019	-0.249	-0.7
ARG	15	0.971	0.019	-0.434	-3.53*
sbPA	13	1	0.021	-1.183	-8.27***
lbPA	11	0.982	0.012	-0.719	-5.15**
lbSA	20	0.853	0.0169	-0.124	1.1

Genetic differentiation among pairwise populations was estimated using F_{ST} and Φ_{ST} (Table 6). The F_{ST} values confirmed the population structure suggested by the nuclear markers (Mantel test, d.f.=6, p=0.008). Populations from the eastern Atlantic (GAL, CEL SCO and ECA) showed low or no significant differentiation, whereas the WNA, ARG and lbSA populations were significantly differentiated from the others and one another. The MAU population was not significantly differentiated from the ECA, ARG populations and either the lbPA or sbPA populations (long-beaked and short-beaked form), although this may be a result of the limited number of samples available from the MAU population. Neither the lbPA nor the sbPA populations showed significant differentiation from the ARG population.

Interestingly, the lbSA and lbPA populations, both classified as the long-beaked form, were significantly differentiated.

Table 6. Genetic differentiation among pairwise populations using mtDNA data. F_{ST} values are reported below the diagonal while Φ_{ST} values are reported above the diagonal. Statistic significance is reported (p<0.05, or * = p<0.001, **= p<0.0001).

	N	GAL	CEL	SCO	ECA	WNA	MAU	ARG	sbPA	lbPA	lbSA
GAL	36		0.005	0.0001	0.077**	0.059*	0.172***	0.132***	0.051**	0.465***	0.099**
CEL	29	0.003		-0.009	0.044*	0.015	0.144**	0.099**	0.032	0.443***	0.058*
SCO	21	-0.011	0.007		0.012	0.037	0.219***	0.124***	0.053*	0.481***	0.083**
ECA	14	-0.003	-0.01	-0.006		0.078*	0.265***	0.127***	0.103***	0.466***	0.104**
WNA	11	0.045**	0.05**	0.053*	0.044*		0.231***	0.029	0.045*	0.54***	0.065
MAU	7	0.04*	0.044*	0.053*	0.028	0.071*		0.25***	0.116**	0.518***	0.204*
ARG	15	0.031**	0.036***	0.043***	0.02*	0.059***	0.037		0.105***	0.506***	0.026
sbPA	14	0.018*	0.022*	0.029*	0.006***	0.044***	0.022	0.014		0.412***	0.107**
lbPA	11	0.026*	0.031*	0.039*	0.015*	0.055***	0.032	0.024	0.009***		0.521***
lbSA	20	0.083***	0.093***	0.102***	0.082***	0.121**	0.105**	0.09***	0.076**	0.087**	

No significant correlation was found between the F_{ST} and Φ_{ST} matrices (Mantel test, d.f.=9, p=0.38). The Φ_{ST} values suggested a different scenario indicating no significant differentiation between the WNA population and two eastern Atlantic populations (ECA and SCO), or the ARG population. Conversely, the MAU population was highly differentiated from all the populations including the two long-beaked populations (lbPA and lbSA). No significant differentiation was observed between the sbPA and the CEL populations. The lbSA did not show significant differentiation when compared with the WNA and ARG populations, while it was highly differentiated from the lbPA population.

The migration rate (M) based on the mtDNA sequences was calculated for the same four populations considered for the microsatellite analysis. The results are reported in Table 4.

Da genetic distance among population was calculated and an unrooted Neighbour-Joining tree was produced (Fig. 4). A separate branch identified the lbPA population. The lbSA population clustered with the MAU population, within the lineage of populations described as the short-beaked form.
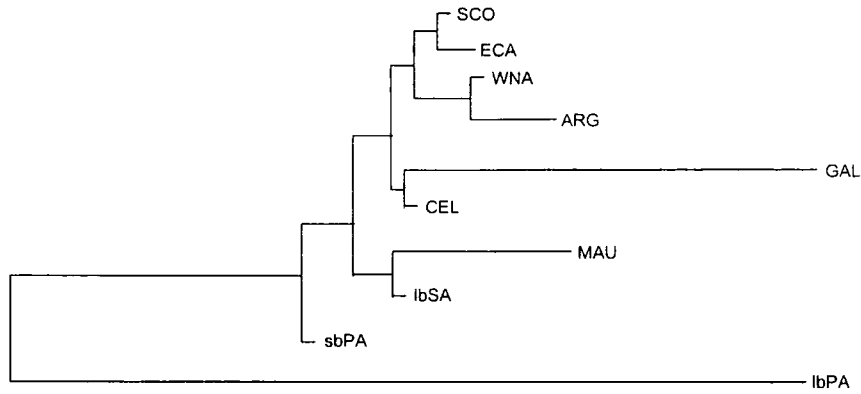
Figure 4. Unrooted Neighbor Joining trees based on Da distances among populations calculated with the mtDNA data. The names of the populations correspond to those given in Table 1.

Rooted (*Stenella attenuata*) Neighbour-Joining and Bayesian trees were reconstructed using all 96 haplotypes (Fig.5). Both methods produced the same phylogeny. The lbPA population diverged from the other haplotypes, but the lbSA population was not monophyletic. Highly supported lineages were observed among other haplotypes, but they did not reflect any geographic clustering.

The spanning network among haplotypes suggested the same pattern (Fig.6a). A high degree of complexity and inter-relatedness among haplotypes was observed when all haplotypes were included in the analysis. No clusters reflected the geographic origin of the haplotypes except for the lbPA population. When only the haplotypes from the eastern Atlantic and WNA populations were considered the structure observed was simpler, but still without reflecting any geographic partition (Fig.6b). The network produced using just the haplotypes from the other populations (ARG, lbSA, MAU sbPA, lbPA) still showed a considerable degree of complexity. Most of the haplotypes were unique. Again, no geographic partitioning was observed, with the exception of the lbPA population.
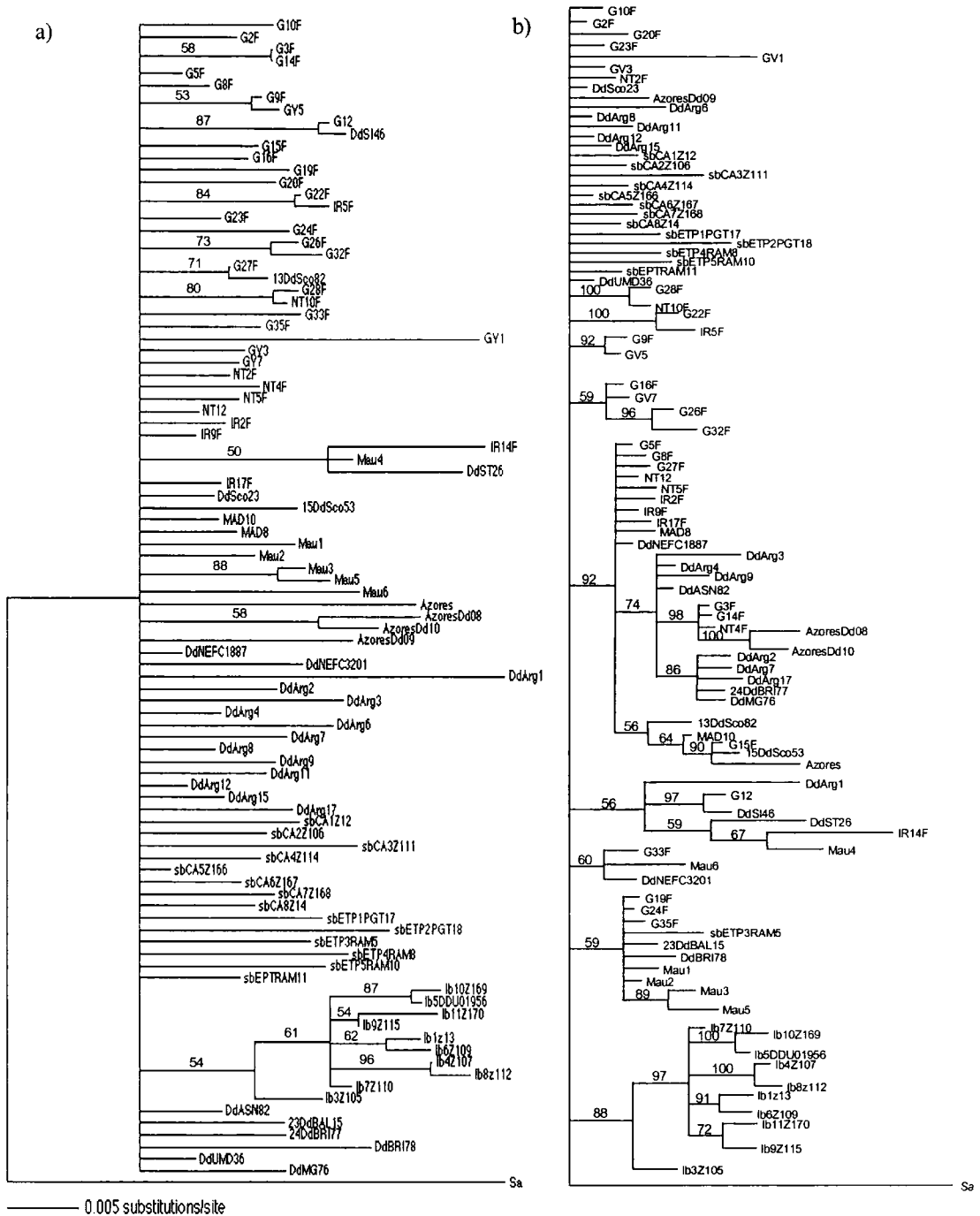
Figure 5. Neighbor-Joining (a) and Bayesian (b) trees illustrating the phylogenetic relationships among 96 mtDNA haplotypes. Bootstrap and Bolean values greater than 50% are indicated. *Stenella attenuata* was used as an outgroup. The names of the haplotypes are reported in Fig. 3.

a)
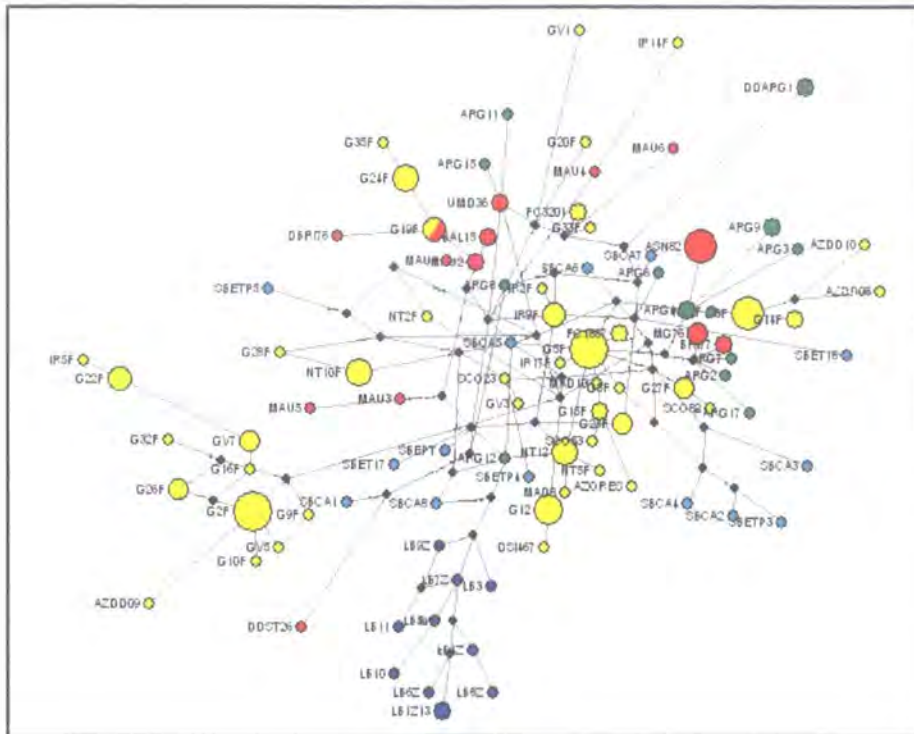
b)   c)

Figure 6. Spanning network among haplotype. a) considering all haplotypes. b) considering only haplotypes from the eastern Atlantic (GAL, CEL, SCO, ECA) and WNA. c) considering only haplotypes from the Pacific Ocean, lbSA, MAU, ARG. MAU = pink, ARG = green, sbPA = light blue, lbPA = blue, lbSA = red, all the other populations in yellow.

Discussion

Our results showed significant genetic differentiation among populations inhabiting different oceans (Indian versus Atlantic), and different sides of the same ocean (eastern Atlantic versus western Atlantic) but no or little differentiation among populations inhabiting the same side of an ocean basin (Table 3 and Table 6). The

Bayesian analysis, based on the individual genotypes and with no assumption on the population structure, suggested similar population partitioning identifying three main clusters corresponding to the lbSA population (Indian Ocean), the WNA/ARG population (western Atlantic Ocean) and the other populations (eastern Atlantic Ocean) (Fig.2). The lbSA population was the most differentiated reflecting the difference in morphology between the long-beaked and short-beaked form.

However, the mtDNA analysis did not support the hypothesis of one single long-beaked lineage worldwide. No shared haplotypes or fixed mutations were observed between the populations described as long-beaked (lbSA, lbPA and MAU) (Fig.3). F_{ST} and Φ_{ST} values between lbPA and lbSA suggested high divergence between these populations, higher than between the lbSA and the short-beaked populations (Table 6) and suggested that the lbPA and lbSA populations may have originated independently in different oceans by different evolutionary events. The lbPA population showed higher Φ_{ST} than F_{ST} values for all pairwise comparisons. A similar trend is observed for the MAU population (putatively long-beaked form), although we cannot exclude the possibility that the limited number of samples may have affected the significance of these values. Higher Φ_{ST} than F_{ST} values are expected when similar haplotypes are associated geographically and the mutation process has been more important relative to other causes of genetic differentiation. Conversely, the lbSA population showed similar Φ_{ST} and F_{ST} suggesting recent population divergence. Recent divergence of the lbSA population is also supported by the microsatellite analysis. The lack of significance for the microsatellite allele size in determining population differentiation suggests that genetic drift is important and the rate of gene flow may be higher relative to the mutation rate.

Differences between the two long-beaked populations were also observed in the genetic diversity. The lbSA population showed reduced gene diversity at both the nuclear and mtDNA level. The lbPA population instead showed high mtDNA gene diversity and significant Fu's F_S values suggesting possible population expansion (Table 5).

Neither did phylogenetic reconstructions support monophyly of the short-beaked and the long-beaked forms worldwide. The Neighbour-Joining tree based on Da distances among populations clustered the lbSA population with the MAU population within the short-beaked lineage, whereas the lbPA population represented

a separated lineage (Fig.4). Both the Bayesian and Neighbour-Joining phylogenetic analyses identified several well-supported clusters but no one reflecting geographic origin, except for the lbPA population that identifies an independent lineage (bootstrap >50%). The network confirmed this scenario showing no lineage sorting except for the lbPA population. In both networks (Fig.6a and 6c) the lbPA haplotypes all originated from a single ancestral haplotype suggesting that the lbPA population originated from a single founder event.

Among the short-beaked form populations, the Bayesian analysis identified two clusters dividing the eastern Atlantic populations (GAL, CEL, SCO, ECA) from the western Atlantic populations (WNA, ARG).

In the eastern Atlantic, nuclear and mtDNA data indicate high gene flow among populations. The low or non-significant F_{ST} values (Table 3 and Table 6) and the high number of shared haplotypes suggest high movements of individuals among these regions. Gene flow also occurs across the Atlantic Ocean, as indicated by the presence of shared haplotypes between the eastern Atlantic and the WNA populations and the relatively low F_{ST} values between the WNA and the eastern Atlantic populations.

The Bayesian analysis clustered the WNA and the ARG populations together. However, significant F_{ST} values and the lack of shared haplotype for the ARG population suggested differentiation between these two populations. A similar degree of genetic differentiation is also observed between the Atlantic populations and the sbPA population (Table 6), indicating that the worldwide population differentiation within the short-beaked form is relatively low, and may be explained by recent population divergence.

Analysis of population dispersal suggested considerable gene flow among the Atlantic populations. Three migrant individuals were identified by the Bayesian analysis and were all apparently migrating from the western Atlantic populations (WNA/ARG) to the eastern Atlantic populations, and included both males and females (Fig.2). Both bi-parental and maternally inherited markers gave similar estimates of migration rates, and for some populations suggested higher movements of females (Table 4). In marine mammals, population structure is generally more evident for mtDNA than nuclear markers, in part due to the different effective population size represented by the two genomes, but likely also due to the more frequent dispersal of males (e.g. Hoelzel *et al.*, 2002). In this case, we found a strong

correlation between nuclear and mtDNA F_{ST} values (Mantel test p=0.008), and observed numerous shared haplotypes among populations (especially among Atlantic populations) suggesting a similar level of dispersal for the two sexes.

Estimates of the migration rates between the IbSA population and other populations were relatively low reflecting higher population isolation. However, higher movement of females towards other areas is also observed for the IbSA population.

In conclusion, we did not find evidence for considering the long-beaked morphotype a single species worldwide. Our data better match the hypothesis of long-beaked populations originated independently in different areas at different times, possibly reflecting adaptation to a particular habitat or specialization for foraging resources. The different pattern observed for different long beaked populations suggest that different evolutionary processes might have been involved in the structuring of different populations. The high morphological variability, often overlapping the long-beaked range, observed in several short-beaked populations (Murphy, 2004, Bell *et al.*, 2002) and our results provide evidence that these may be source populations for the long-beaked populations.

The short-beaked morphotype shows low genetic differentiation across oceans suggesting high gene flow, or in some cases recent population divergence. Dispersal patterns suggest that female phylopatry is not a strategy adopted by the common dolphins, but that both sexes move together.

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CHAPTER 6

Conservation genetics of the short-beaked common dolphin (*Delphinus delphis*) in the Mediterranean Sea and in the eastern North Atlantic Ocean

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Abstract

The common dolphin Mediterranean population has recently been listed as 'endangered' in the IUCN Red list. This is due to the population's drastic decline since the middle of the twentieth century, especially in the central region of the basin. However, little is know about the structure and identity of this population. We analysed 118 samples from the Black Sea, Mediterranean Sea and eastern North Atlantic using nuclear and mtDNA markers. We found population differentiation across the basin between the eastern and the western Mediterranean, and could not exclude further population structure in the central area of the basin. Such structure matched the different distribution pattern and the different habitat use exhibited in the eastern and the western part of the Mediterranean Sea. These regions are defined by different oceanographic characteristics. Moreover, nuclear and mtDNA data suggested similar dispersal for males and females. However, evidence for directional migration of females was observed from the easternmost marginal populations towards the Atlantic populations. These data suggest that adaptation to different habitat may have shaped the population structure observed.

Keywords: common dolphin, population genetics, Mediterranean Sea, sex-biased dispersal, conservation.

Introduction

The status of the common dolphin (*Delphinus delphis*) population in the Mediterranean Sea has been a concern for many years. In 2003 the Mediterranean common dolphin 'subpopulation' was listed as endangered in the IUCN Red List of Threatened Animals, based on criterion A2, which refers to a "50% decline in abundance over the last three generations". It is also listed in Appendix II of the Washington Convention (1973), in Appendix II of the Bonn Convention (1983), in Appendix II of the Bern Convention (1986), in and in Annex IV of the European Union Habitats Directive (1992). According to ACCOBAMS (Agreement for the Conservation of Cetaceans in the Black Sea, Mediterranean Sea and Atlantic contiguous waters) this species should be considered endangered.

The common dolphin shows an extremely wide distribution in all oceans, from warm temperate to tropical waters. However, in the Mediterranean Sea it is disappearing from most of its historical habitat range, principally in the central region of the basin (Ligurian Sea, Gulf of Lion, Tyrrenian and Adriatic Sea), although isolated populations are still monitored in the Alboran Sea, northern Sardinia, south Tyrrhenian Sea, Malta, and Ionian Sea (Bearzi *et al.*, 2003). Literature references, photographic documentation and osteological collections indicate large common dolphin populations in these regions until the middle of the 20th century (Duguy & Cyrus, 1973; Casinos & Vericad, 1976; Poggi, 1986; Cagnolaro, 1994; Bearzi *et al.*, 2004). The causes of this sudden decline are not fully understood and this may be an ongoing trend (Bearzi *et al.*, 2003).

The Mediterranean Sea is an enclosed basin where the intensity of human activities has significantly impacted on the marine environment especially along the coastal areas. The most probable factors implicated in the decline of this species have been identified as global environmental changes, prey depletion, xenobiotic contamination and direct takes and bycatch (for a detailed review of these factors, see Bearzi *et al.*, 2003).

The distribution pattern of the common dolphin differs between the western and the eastern Mediterranean Sea. In the western area (Alboran Sea, Algeria and Balearic Sea), the species is recorded at all depths, but is mainly oceanic, inhabiting primarily waters beyond the continental shelf (>150 m depth) (Viale & Frontier, 1994; Forcada & Hammond, 1998; Gannier, 1995; Cañadas *et al.*, 2002; Cañadas *et*

al., submitted). In this region it frequently forms mixed groups with striped dolphins (Cañadas *et al.*, 2002). Conversely, in the Adriatic Sea and Ionian Sea it has been observed primarily in neritic areas where it often occurs sympatrically with the bottlenose dolphin (Bearzi, 2003; Politi *et al.*, 1992; Frantzis, Pers. com.). In the Aegean Sea, it is also observed mainly in coastal shallow waters over the continental shelf (Frantzis *et al.* 2003). In the enclosed and deep Gulf of Korianthiakos (Greece) this species is oceanic, but it has always been recorded in mixed groups with striped and Risso's dolphins and never on its own (Frantzis & Herzing 2002).

The density of common dolphins also seems to vary widely across the Mediterranean Sea. In the Alboran Sea, the species is abundant, estimated at 14,736 individuals, (95% CI = 6,923-31,366) according to Forcada and Hammond (1998). On the other hand, although no abundance estimates exist for the rest of the Mediterranean, the data available indicate that only a few hundred animals inhabit Greek waters (Ionian and Aegean Seas), and some coastal areas of the Thyrrenian Sea (Bearzi *et al.*, 2003; Frantzis *et al.*, 2003). In the Adriatic Sea and Israel, common dolphins are very rare (Bearzi *et al.*, 2004, Kerem, Pers. com.). Increasing concern has arisen from a closely monitored population that used to inhabit the waters surrounding the island of Kalamos (Ionian Sea). This population has gone through a very rapid decline during the last 7- 8 years (Bearzi, 2003). The demographic trend of this population mirrors the situation in the central Mediterranean, and suggests that the causes of the decline in the common dolphin population in the Mediterranean Sea may still be active.

Given the pace of this decline, it is fundamental to determine the population identity of this species in this area, as baseline information required for the development of an adequate, and urgent, conservation plan, as recommended by ACCOBAMS. For this purpose, in this study we analysed samples from the Mediterranean Sea and we compared them with samples from the Eastern North Atlantic and from the Black Sea. Our aim is to identify the population boundaries across this range and within the Mediterranean Sea, assessing (1) whether the Mediterranean common dolphin is a homogeneous population, (2) the relationship with the contiguous Eastern North Atlantic populations, and (3) the level of gene flow between Mediterranean and Atlantic populations. We also attempt to infer the identity of the putative population in the Central Mediterranean by assigning samples collected at different stages in the population decline.

Materials and Methods

Sample collection and DNA extraction

A total of 118 samples were analysed. Samples were from stranded animals or biopsy sampling. Samples were from the Black Sea (5), the Ionian Sea (22 – these samples were mainly from a resident coastal population inhabiting the waters around the island of Kalamos, Greece), from the Alboran Sea (34), from Portugal (16) and from Galicia (31). Additionally, samples from different areas of the Mediterranean Sea were analysed: one sample from the Aegean Sea, one sample from Sicily, one sample from Tyrrenian Sea (western coast of Italy), two samples from the eastern coast of France, two samples from Valencia, and three samples from Algeria (eastern Oran) (Fig.1). One sample from France was a museum specimen from the beginning of the twentieth century.
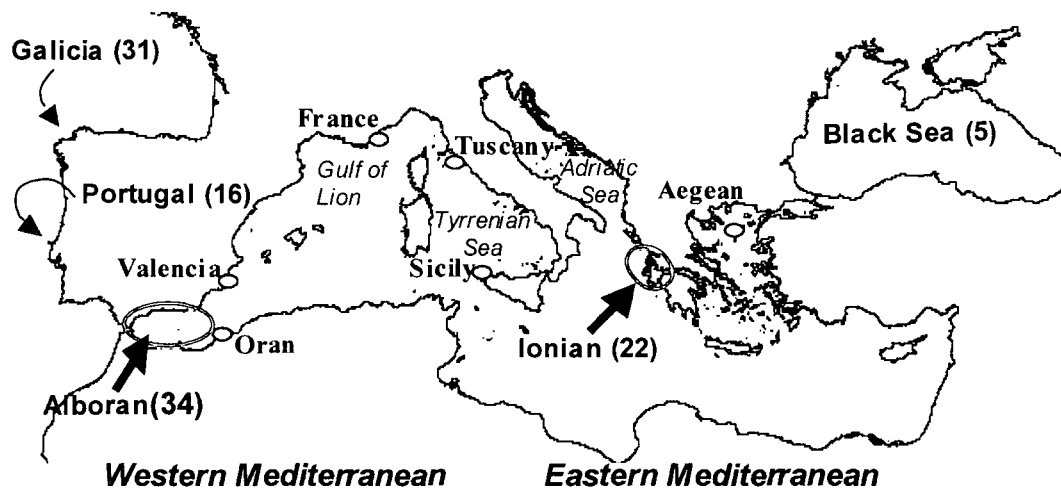
Figure 1. Map of the Mediterranean and Black Sea. Red dots indicate the samples from the central Mediterranean Sea and Aegean Sea. Green circles indicate the Ionian and Alboran Sea populations.

DNA was extracted from tissue samples preserved in salt saturated 20%DMSO by a standard phenol/chloroform extraction method (Hoelzel, 1998). DNA was extracted from bone samples using QIAgen PCR purification columns after grinding 100mg of bone and digesting it at 37°C for 48 hours in 1ml of digestion buffer (0.01M TRIS, 0.01M NaCl, 1% SDS, 2mg/ml proteinase K, 0.01 PTB). To avoid contamination the extraction and the analysis of the bone specimens were conducted in a different laboratory where no cetacean DNA had ever been manipulated before. An extraction including everything but tissue was undertaken for all analyses as a negative control.

Sex determination

Individuals whose gender was unknown were sexed by amplifying portions of the genes ZFX and ZFY as described in Bérubé and Palsbøll (1996).

Microsatellite analysis

Samples were genotyped at 9 microsatellites loci: KWM1b, KWM2a, KWM2b, KWM9b, KWM12a were derived from *Orcinus orca* (Hoelzel *et al*., 1998), EV37Mn from *Megaptera novaeangliae* (Valsecchi & Amos, 1996), TexVet5, TexVet7 and D08 from *Tursiops truncatus* (Rooney *et al.* 1999, Shinohara *et al*.1997). PCR conditions were after Natoli *et al*. (Chapter 5). Amplified DNA was analysed for length variation on 6% polyacrylammide denaturing gels using fluorescent imagining on an automated ABI PRISM 377 DNA sequencer, after incorporation of 1/10 fluorescent labelled primer. An internal standard marker (Genescan-500 ROX, Applied Biosystems) was used to determine the allele sizes.

The level of genetic diversity was estimated as observed heterozygosity (H_o), expected heterozygosity (H_e), and allelic richness. Allelic richness controls for variation in sample size by a rarefaction method, and was calculated using the program FSTAT 2.9.3 (Goudet, 2001). Evaluation of possible deviations from Hardy Weinberg (overall deviation, heterozygote deficiency and heterozygote excess) was performed using Fisher's exact test and the Markov chain method (dememorization number, number of batches, iteration per batch set at 1,000, Bonferroni correction applied). The level of intrapopulation differentiation was estimated as F_{IS}. These analyses were performed using GENEPOP 3.1d (Raymond & Rousset, 1995a,b).

The level of differentiation among populations was estimated as F_{ST} (Weir & Cockerham 1984) using the program ARLEQUIN 2.0 (Schneider *et al.,* 1999).

The most probable number of putative populations (K) that best explains the pattern of genetic variability was estimated using the program STRUCTURE 2.1 (Pritchard *et al*., 2000). We assumed the admixture model and we performed the analysis considering the correlated allele frequency model. Burning length and length of simulation was set at 100,000 and 1,000,000 repetitions, respectively. To test the convergence of the priors and the appropriateness of the chosen burn in length and simulation length, we ran a series of independent runs for each value of K (for 1<K>4) as suggested by Pritchard *et al*. (2000). We tested whether any particular individual was an immigrant or had an immigrant ancestor, by using the model with

prior population information, subdividing the individuals into K populations, according to the results of the previous analysis. We assumed v (migration rate) = 0.05 and 0.1, and testing for 0<number of generations (G)>2.

An asymmetric estimate of the migration rate ($M=4N_em$) between pairwise populations, based on microsatellite and mtDNA data, was calculated using MIGRATE 1.7.3 (Beerli, 2002). The length of the runs was optimised for both markers (acceptance-rejection > 2%, R<1.2). Initial runs were set estimating θ and M with F_{ST} and allowing M to be asymmetric. Reruns were set using the parameter estimated with the first run and lengthening the MCMC chains. In order to verify the result a final run was set using longer chains. For comparison the migration rate was also calculated by hand according to $F_{ST}=1/(4Nm+1)$.

Possible first generation migrants were assessed using GENECLASS 2.0b (Piry S, *et al.*, submitted). The program was set on the Rannala and Mountain criterion (1997), and on the simulation algorithm described in Paetkau *et al.* (2003) (10,000 repetitions). The threshold was set at 0.01. Three populations (Black Sea, Ionian, Alboran-Atlantic populations) were considered.

GENECLASS 2.0b was also used to assign individuals from unknown populations (Aegean, Tyrrenian, Valencia and Algeria). The Ionian and Alboran Sea were used as reference populations. The Bayesian method (Rannala and Mountain, 1997) was used as criteria of computation. The individual's scores were calculated as the likelihood of an individual to belong to a population divided by the sum of the likelihoods of that individual for all populations. The assignment threshold was set to 0.01. The computation of probability was performed using the MCMC resampling method (number of simulated individuals set to 10,000; α set to 0.01) (Paetkau, 2003).

Genetic distances between individuals were estimated using Nei's Da genetic distance (Nei *et al.*, 1983). Calculations were performed using the programme MicroSatellite Analyser (MSA) (Dieringer and Schlotterer, 2002).

Multidimensional scaling analysis was performed using the program *XL*-Stat Pro 6.0 based on a matrix of Da distances among pairwise individuals.

Sex-biased dispersal was tested using the program FSTAT 2.9.3 (Goudet, 2001). Only adult individuals (a total of: 42 females and 55 males) were considered for this analysis.

mtDNA analysis

A total of 114 samples were sequenced for 428 bps at the 5' end of the mtDNA control region and compared with 4 sequences already published (from the Black Sea, Rosel *et al.*, 1994). In total 118 sequences were considered for the data analysis.

The mitochondrial DNA control region was amplified either with universal primers MTCRf (5'-TTC CCC GGT GTA AAC C) and MTCRr (5'-ATT TTC AGT GTC TTG CTT T) after Hoelzel (1998), or with the primers 5'-ACA CCA GTC TTG TAA ACC-3' and 5'-TAC CAA ATG TAT GAA ACC TCA G-3' after Rosel *et al.* (1994). The PCR reaction conditions and PCR cycling profile were as reported in Chapter 5. PCR products were purified with QIAgen PCR purification columns and sequenced directly using the ABI dye-terminator method.

Mitochondrial DNA from the French coast bone samples was amplified using two sets of primers designed in order to amplify two overlapping portions of the control region of approximately 200bps each (Dmtcrf: 5'-TTA GTC TCT CCT TGT AAA T-3' and Dmtcrr: 5'-GGT GAT TAA GCT CGT GAT-3' (Nichols, 2004); MTCRf and mtancr: 5'-AAA ATA AAT GAA TGC ACA ATA-3'). The PCR reaction conditions and PCR cycling profile were as reported in Chapter 5. A total of 357 bps were amplified. Sequence alignment was performed using ClustalX (Thompson *et al.*, 1997).

The degree of differentiation (F_{ST}), the nucleotide diversity (π), Tajima's D and Fu's F_S were estimated using ARLEQUIN 2.0 (Schneider *et al.*, 1999).

A median-joining network was generated to infer phylogenetic relationships among the mtDNA haplotypes, using the program NETWORK 2.0 (Bandelt *et al.* 1999; www.fluxus-engineering.com).

Results

Assessing genetic variation and population differentiation

Samples were divided in five populations based on their geographic origins. Considering the microsatellite data, the populations were tested for deviation from the HW equilibrium. Observed and expected heterozygosities were calculated at each locus for each population (Table 1).

Table 1. Genetic variation at each locus for each population. The number of individuals analysed for each population is indicated below the population name. BS stands for Black Sea population. The number of different alleles, number of private alleles (in parenthesis) and allelic richness (in squared brackets), heterozygosity observed (Ho), and heterozygosity expected (He) are reported. The respective averages (standard deviation in parenthesis) are reported in the last rows. One asterisk indicates the loci that showed significant deviation from the HW equilibrium (p<0.05); two asterisks indicate those loci still significant after Bonferroni correction (p<0.001).

		Populations				
		BS	**Ionian**	**Alboran**	**Portugal**	**Galicia**
		N=5	N=22	N=34	N=16	N=31
KWM1b	N. Alleles 4	2 [1.98]	2 [1.59]	4[1.55]	3 [2.04]	3 [1.82]
	Ho	0.5	0.191	0.147	0.186	0.226
	He	0.53	0.219	0.142	0.375	0.257
KWM2a	N. Alleles 16	4 (1) [3.58]	6 [4.14]	14 (2) [5.59]	10 [5.13]	12 (1) [5.6]
	Ho	0.667	0.818	0.882	0.688	0.839
	He	0.636	0.801	0.887	0.871	0.887
KWM2b	N. Alleles 8	5 [4.56]	6 [3.77]	8 (1) [4.85]	6 [4.61]	7 [4.62]
	Ho	1	0.773	0.882	0.813	0.774
	He	0.788	0.744	0.849	0.829	0.83
KWM9b	N. Alleles 12	4 [3.78]	8 [4.35]	9 [4.87]	7 [4.43]	12 [5.52]
	Ho	0.333*	0.864	0.879	0.875	0.936
	He	0.788	0.784	0.846	0.815	0.886
KWM12a	N. Alleles 10	4 [3.6]	5 [3.98]	9 (1) [4.75]	7 [4.69]	9 (1) [4.68]
	Ho	0.667	0.772	0.765	0.625*	0.871
	He	0.682	0.760	0.823	0.823	0.796
EV37Mn	N. Alleles 24	6 [5.36]	11 [4.87]	15 (1) [5.75]	14 [6.0]	19 (3) [6.1]
	Ho	0.5	0.818	0.941	1	0.968
	He	0.924	0.825	0.899	0.913	0.918
TexVet5	N. Alleles 11	2 [2]	7 [4.59]	10 [4.98]	8 [4.64]	10 [4.93]
	Ho	0.2	0.682	0.656**	0.786	0.5**
	He	0.467	0.832	0.855	0.841	0.854
TexVet7	N. Alleles 7	2 [1.8]	3 [2.3]	5 (1) [3.52]	5 (1) [3.89]	5 (1) [3.7]
	Ho	0.333	0.364	0.879	0.688	0.677
	He	0.439	0.503	0.725	0.788	0.742
D08	N. Alleles 13	3 [2.6]	7 [4.16]	10 [5.05]	10 [5.16]	12 (3) [5.04]
	Ho	0.5	0.682	0.735	0.875	0.645**
	He	0.561	0.793	0.872	0.875	0.845
Average (SD)	N. Alleles	3.6 (1.4) [3.25 (1.24)]	6.1 (2.7) [3.75 (1.09)]	9.33 (3.6) [4.55 (1.29)]	7.8 (3.2) [4.51 (1.09)]	9.89 (4.7) [4.67 (1.27)]
	Ho	0.52 (0.24)	0.66 (0.23)	0.75 (0.24)	0.73 (0.23)	0.72 (0.23)
	He	0.65 (0.16)	0.7 (0.2)	0.77 (0.24)	0.79 (0.16)	0.78 (0.21)

The Alboran and the Galician population deviated significantly from the HW equilibrium at one and two loci respectively (p<0.001, Bonferroni correction applied). Average allelic richness, observed and expected heterozygosities were lowest for the Black Sea and the Ionian populations. However, the limited number of samples for the Black Sea population should be taken into account when considering these results.

The Ionian population showed lower allelic richness and expected heterozygosity when compared with the Alboran and the Atlantic populations,

whereas the Alboran population showed similar values to those of the Atlantic populations.

For mtDNA, forty-five different haplotypes were observed based on 42 variable sites (Fig. 2).

```
               111111 1111111122 2222333333 3333333334 44    BS  Ion Tyr Alb  Port Gal
             2356113333 3445777934 7779000011 2224557891 11    9   22  5   34   17   30
             9686050234 7356678414 1341568909 0792054361 78   ----------------------------
#BSDD4   -CAAGTCCAT ATACATTTTT CTCATCTTCC TTGAACCTTC TC    1   6       9    2    2
#BSV1    -....C.... .........C .......C.. C......... C.    3   3   1   2    2    2
#BSV2    -....C.... .........C .......C.. C.....-... CT    1
#BSG11   -......... .........C .......... .......... ..    1
#BSDd5   -......... .......... .......T.. .......... ..    1
#DdN423  -......... .......... ...G...... .......... ..    1
#DdN432  -......... .......... .....T.... .......... ..    1
#DELC1   -......... .......... .......... ...T..... ..        3
#DEL2    -....ACT... ...T..A..C ..AG...CT. C.T....... ..        5
#Dd1.02  -....C.... .........C .......C.. C......... CT        4
#Dd3.02  A....C.... ....G...CC ..G.....T. C...G..... ..    1   1   1         1
#Bom2    A....C.... ......A..C .G.C....T ...T...---- --           1
#Bom3    -......... ......G..C .G......T ........---- --           1
#ALB1    -......... .......... .......C.. .......... C.                 1
#ALB11   -....C.... .........C .......C.T C......... C.        1   11
#ALB16   -....C.... ........CC T.A....C.. C......C.. C.                  2
#ALB17   A....C.... ......A..C .G.C....T ..T.G..... ..                  2
#Alb24   -....CT... .........C .......... C......... C.                  1   1    1
#Alb25   -....C...C .........C ..G......T C.A....... C.                  1
#Alb28   -....C.... ....G....C ..G.....T. C...G..... C.                  1
#ALB3    -....C...C ..G......C ..G......T CC........ C.                  1
#ALB4    -....C.... .........C .......C.. CC........ C.                  1
#BG10F   -......... .......... ......C... .......... ..                  1
#P8F     -....C...C .........C ..G...CC.. C.AG...... C.                       2    1
#P10F    -....C.... ........CC .......C.. C......... C.                       1    1
#P11F    A......... .......... .......... .......... ..                       1    1
#P14F    -.C..C.... .........C .C.....C.. C......... C.                       1    1
#P16F    -....C...C .......C.C ..GG.....T .......... C.                       1
#P17F    -....C.... ....G...CC ..G.....T. C...G..C.. ..                       2
#P19F    -....C.... .........C .......C.. C.......A C.                        1
#P20F    -....C...C .........C ..G......T .......... C.                       1    4
#P22F    -....C.... G........C .......CT. C......... C.                       1
#P24F    -......... .........C .......C.. .......... ..                       1
#G-12    A....C.... ......A..C .G.C..... ..T.G...C. ..                             1
#G-34    A....C.... ......A..C .G.C..... ..T.G..... ..                             2
#G10F    -....C..GC .........C ..G......T .......... C.                             1
#G16F    -....C...C .........C ..G........ ..A....C.. CT                            1
#G19F    -....C.... ....G...CC ..G.....T. C...G..... ..                             2
#G20F    -....C.... ....C...C ..G...C.T. .......... ..                             1
#G23F    -....C.... .........C ..A....CTT C......... ..                             1
#G26F    -....C...C ..G......C ..G......T C.A....... C.                             1
#G28F    -..G.C.... .C.......C T.G....C.. ........C.. C.                            1
#G32F    -....C.T.C ..G......C ..G....... C.A....... C.                             1
#G33F    -....C..G. .........C ..G..TCCT. .......... C.                             1
#G35F    A....C.... ....G....C ..G.....T. C...G..... ..                             1
#G5F     -....C.... .........C .......C.. C......C.. ..                             1
#G9F     -T...C...C .........C ..G....... .......... C.                             1
```

Figure 2. Polymorphic sites among 45 haplotypes are shown (left). Haplotype frequencies (right) were reported for each haplotype in each population. BS=Black Sea, Ion=Ionian, Tyr=Tyrrenian Alb=Alboran, Port=Portugal, Gal=Galicia. The total number of haplotype analysed for each population is reported below the population code.

Two common haplotypes were shared among all populations. Overall gene and nucleotide diversities were high at 0.943 and 0.015, respectively. The Alboran population showed the lowest gene diversity, whereas the Black Sea population showed the lowest nucleotide diversity (Table 2). Fu's Fs test of selective neutrality was found highly significant for the Galician population (Fs=-11.88, p<0.02),

suggesting possible demographic population expansion. However, Tajima's D test was negative but not significant (p=0.28 Beta distribution approximation).

Table 2. Genetic diversity (Gene Div.), nucleotide diversity (Nucl. Div.), and the values of tests for neutrality Tajima'D and Fu's Fs are reported for each population.

	N	Gene Div.	Nucl. Div.	Tajima' D	Fu'Fs
Black Sea	9	0.917	0.009	0.494	-2.0
Ionian	22	0.84	0.015	1.08	4.19
Alboran	34	0.832	0.012	-0.138	-1.29
Portugal	17	0.971	0.014	-0.447	-4.157*
Galicia	30	0.977	0.016	-0.653	-11.88***

Genetic differentiation among pairwise populations was estimated using F_{ST} (Table 3). Based on the microsatellite data, the Black Sea and Ionian populations were significantly differentiated from one another and from all other populations. Conversely, no genetic significant differentiation was detected among Alboran, Gibraltar and the Atlantic populations. The mtDNA analysis confirmed the differentiation observed between the Ionian and Alboran populations, but also indicated significant differentiation between the Alboran population and the Atlantic populations (Galicia and Portugal). Instead, the Black Sea population was significantly differentiated only from the Alboran population. Again, the limited number of samples for this population should be taken into account when considering these results.

Table 3. Pairwise population differentiation values expressed as F_{ST} based on microsatellite data (below diagonal) and mtDNA haplotype frequencies (above diagonal). Sample size for the microsatellites for each population is reported in the third coloumn. Sample size for the mtDNA data is reported in the third row. Statistic significance is reported as follows: * = p<0.05, ** = p<0.01, *** = p<0.001.

	Pop		Black Sea	Ionian	Alboran	Portugal	Galicia
			mitochondrial DNA				
		N	9	22	34	17	30
Microsatellites	**Black Sea**	5		0.053	0.086*	0.003	0.021
	Ionian	22	0.099***		0.09**	0.05*	0.063***
	Alboran	34	0.102***	0.053***		0.065*	0.074***
	Portugal	16	0.096***	0.056***	-0.002		-0.009
	Galicia	31	0.097***	0.052***	-0.001	-0.003	

The Bayesian analysis, based on the microsatellite data, was used as an alternative approach to test the population structure suggested by the F_{ST} values. The number of populations (K) that best explained the genetic variability observed across

our samples was 2, supporting the differentiation between the eastern and the western Mediterranean populations suggested by the F_{ST} analysis (Fig. 3). Consistency among different runs was observed for the estimate of P(X/K) and the prior α, indicating that the burn-in length and the length of the runs were appropriate.
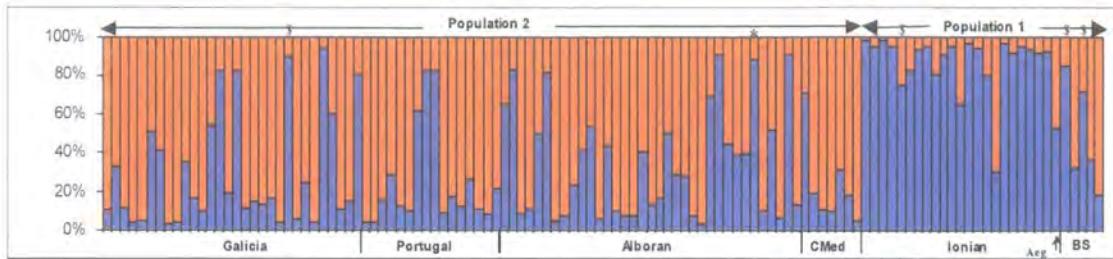

Figure 3. Estimated proportion of the coefficient of admixture of each individual's genome that originated from population *k*, for K=2. Each individual is represented by a column. The asterisk indicated the individual identified as migrant by Structure and Geneclass. The $ indicated the individuals identified as migrants by Geneclass.

The spanning network was drawn including the haplotypes of the nine individuals from different areas of the Mediterranean Sea. No cluster reflected geographic origin (Fig. 4).
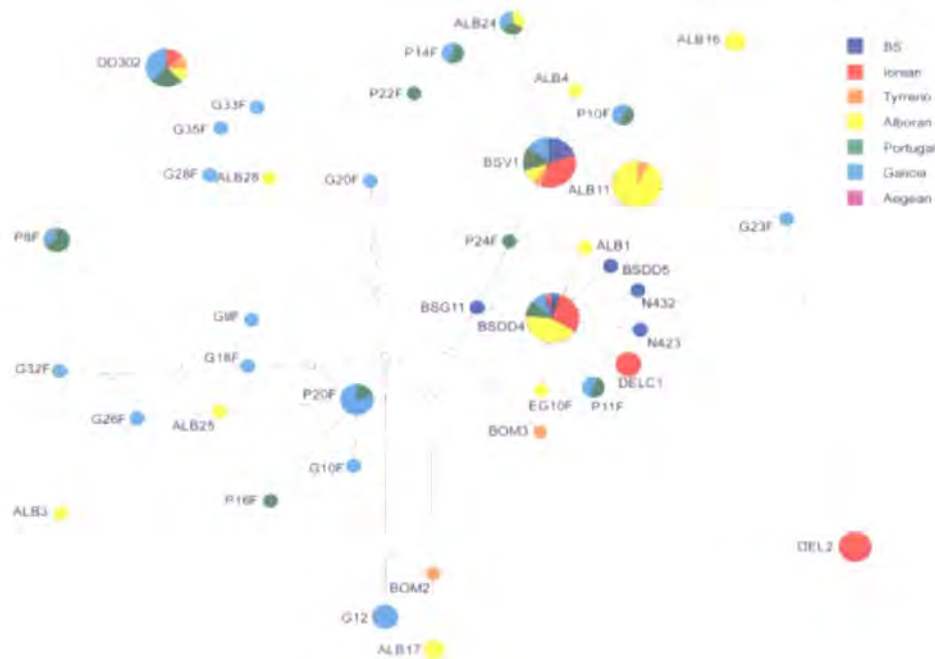

Figure 4. Minimum spanning network among haplotypes. The size of the circles is proportional to the total number of haplotypes observed. Sectors are proportional to the number of each haplotype observed in each population. Populations are identified as follows: Black Sea population in blue, Ionian population in red, Alboran population in yellow, Portugal in green and Galicia in light blue. Orange indicates the haplotypes from the Tyrrenian Sea and pink the haplotype from the Aegean Sea. White circles indicate ancestral extinct haplotypes. Haplotypes names are the same as in Fig.2.

Migration rate, migrants and sex biased dispersal

We used two different programmes to detect possible migrants: STRUCTURE 2.1 and GENECLASS 2.0b. STRUCTURE identified one possible first generation immigrant from the eastern Mediterranean population to the Alboran population at both the values of v=0.1 and v=0.05. GENECLASS suggested four possible first generation immigrants including the individual identified by STRUCTURE 2.1 (Fig. 1).

Classic F_{ST} based migration rates and asymmetric migration rates were calculated for each pair of contiguous populations with both nuclear and mtDNA markers (Table 4). Values based on the microsatellite markers did not show any directional movement, while those based on the mtDNA markers suggested directional migration from east to west for all neighbouring pairs of populations.

Table 4. Estimate of the migration rate (M) between contiguous populations, based on the microsatellite and mtDNA data. Nm columns refer to the values calculated according F_{ST}=1/(4Nm+1). The other columns refer to the asymmetrical migration rate calculated using a maximum likelihood method (MIGRATE): 1,2 stands for: migration from population 1 to population two; 2,1 stands for: migration from population 2 to population 1. The interval confidence (95% c.i.) is also reported.

Population	microsatellites (bi-parental)				mtDNA (maternally inherited)			
	Nm	1,2	2,1	95% c.i.	Nm	1,2	2,1	95%c.i.
1 BlackSea	2.280	--	10.960	9.93-12.06	8.930	--	0.911	0.797-3.282
2 Ionian		4.530	--	4.13-4.97		6.074	--	2.436-10.34
1 Ionian	4.467	--	7.534	6.9-8.57	5.060	--	2.570	1.81-3.46
2 Alboran		8.538	--	7.95-9.15		34.598	--	27.21-43.37
1 Alboran	inf	--	13.512	12.65-14.41	7.190	--	6.070	3.21-7.41
2 Portugal		16.718	--	15.75-17-70		87.230	--	25.65-105.86
1 Portugal	inf	--	9.58	8.77-10.32	inf	--	0.158	0.138-0.795
2 Galicia		13.46	--	12.58-14.39		93.145	--	70.89-171.85

Sex-biased dispersal was tested among populations. Although F_{IS} was significantly lower in females than in males, the other parameters analysed (relatedness index, mean assignment test, Ho, and He) did not show any significant difference between males and females (Table 5).

Table 5. Different tests to assess sex-biased dispersal and respective p-values are reported (calculated after 1000 randomizations). Relat = coefficient of relatedness, Hs = within group gene diversity, MeanAss = mean assignment test, VarAss = variance of the Assignment test.

	N	Fis	Fst	Relat	Mean.Ass.	Var.ass.	Ho	Hs
F	42	0.013	0.057	0.107	-0.186	8.495	0.738	0.747
M	56	0.084	0.049	0.086	0.139	11.285	0.682	0.745
overall		0.05	0.062	0.111			0.706	0.743
p-value		0.032	0.57	0.45	0.58	0.39	0.074	0.923

Population assignment of individuals

We attempted to assess the most probable source populations for the nine unassigned samples collected from Algeria, the Tyrrenian Sea, and the Aegean Sea).

An assignment test was performed using the Ionian and Alboran Seas as reference populations. One sample from Oran and one from France were assigned to the Ionian population, according to the likelihood values and the associated probability values. According to the likelihood values, the other samples were assigned to the Alboran population. However, not all the assignments were supported by the associated probability values. For the samples from the Aegean and Tuscany, one sample from Valencia and one from Oran the probability of belonging to any of the populations were equal to zero (Table 6).

Table 6. Assignment test for individuals from the central Mediterranean using GENECLASS 2.01. Pop1 = Ionian population, Pop2 = Alboran population. The likelihood scores (score%) and likelihood values to both populations are reported. The associated probabilities for each population calculated using a Montecarlo Chain Method (10,000 replications) are reported on the right of the table. The number of loci used to assign each individual is reported. The 'code' refers to the same individuals in Fig.5. The asterisk indicates a pre-decline sample.

Code	Origin	Likelihood				Pop1	Pop2	MCMC		N. Loci
		Ass to pop	score%	Ass to pop	score%	Pop1	Pop2	Pop1	Pop2	N. Loci
1	Aegean	Alboran	99.994	Ionian	0.006	27.411	23.191	0.000	0.000	9
2	Tuscany	Alboran	100.000	Ionian	0.000	27.059	21.726	0.000	0.000	9
3	Sicily	Alboran	99.979	Ionian	0.021	25.455	21.782	0.144	0.183	8
4	France*	Ionian	99.943	Alboran	0.057	13.501	16.745	0.493	0.047	5
5a	Valencia	Alboran	99.983	Ionian	0.017	23.619	19.839	0.000	0.002	9
5b	Valencia	Alboran	100.000	Ionian	0.000	19.967	12.987	0.000	0.285	9
6a	Oran	Alboran	100.000	Ionian	0.000	17.964	11.498	0.080	0.555	5
6b	Oran	Alboran	100.000	Ionian	0.000	28.181	22.639	0.000	0.000	9
6c	Oran	Ionian	93.469	Alboran	6.531	11.255	12.411	0.638	0.418	5

Multidimensional scaling analysis was used as an alternative method to assign the aforementioned individuals. It supported the differentiation between the Ionian population and Alboran population and clustered the nine unassigned samples as shown in Fig. 5.
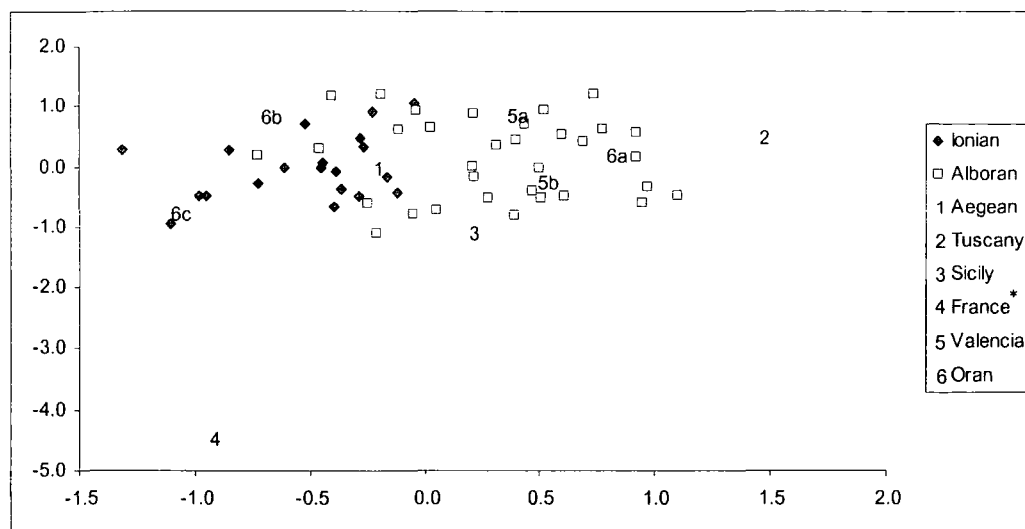
Figure 5. Multidimesional scaling analysis based on Da distance between pairwise individuals. Axes x and y represented dimension 1 and 2 respectively. Individuals from the Ionian population are in black. Individuals from the Alboran population are in open squares. Numbers indicate the individuals assigned and correspond to those reported in Table 6. The asterisk indicates the sample from the pre-decline period.

Discussion

Our first objective was to test for evidence of subpopulation structure in Mediterranean Sea. The Bayesian analysis, based on individual genotypes (STRUCTURE), suggested differentiation within the Mediterranean between the Ionian and Alboran populations. Both nuclear and mtDNA F_{ST} analyses supported this result showing clear differentiation between the Ionian and Alboran populations (Table 2). Significant differentiation was also detected at the nuclear level for the Black Sea population, but not confirmed by the Bayesian analysis or the mtDNA data, although this may be a result of the limited number of samples available from the Black Sea population.

Considering the relative proximity of the Ionian and Alboran populations, such marked differentiation was unexpected. The common dolphin is a highly mobile species capable of long distance dispersion, confirmed by the high level of panmixia (lack of strong population structure) observed world-wide (see Chapter 5). Therefore, it is reasonable that the geographic distance is probably not responsible for the limited gene flow observed within the Mediterranean Sea. Moreover, if considered in a worldwide contest, the F_{ST} values observed between the two Mediterranean populations are considerably high. Common dolphin populations from different sides of the Atlantic Ocean show similar or even lower genetic differentiation (F_{ST} values

based on microsatellites were between 0.012 and 0.045; F_{ST} values based on mtDNA were between 0.028 and 0.059).

Differentiation between western and eastern Mediterranean populations has been observed in other marine species such as the common sole (*Solea vulgaris*; Guarniero *et al.*, 2002) and the sea bass (*Dicentrarchus labrax*; Bahri-Sfar *et al.*, 2000). A strong population boundary has also been observed in the bottlenose dolphin (*Tursiops truncatus*) between the western and the eastern Mediterranean populations (Chapter 3). Adaptation to different habitats through specialisation in different foraging behaviours was suggested as a possible factor for the differentiation of the populations in this region (Chapter 3). The fact that the common dolphin shows a different distribution preferring deep oceanic waters in the western Mediterranean and neritic waters in the eastern Mediterranean suggest adaptation to the exploitation of different resources and is consistent with this hypothesis.

Our second objective was to assess the relationship between the Mediterranean and the Atlantic populations. The Alboran population was not differentiated at nuclear markers from the Atlantic populations, though mtDNA analysis showed significant differentiation.

Despite the presence of the Strait of Gibraltar, the oceanographic characteristics of the Alboran Sea are similar to those of the eastern North Atlantic Ocean. The Almeria-Oran front situated 350 km within the Mediterranean Sea represents the actual shift between oceanic and Mediterranean waters. Common dolphins in both these areas are generally observed in open waters off the continental shelf (Cañadas *et al.*, 2003, Lopez *et al.*, 2004). It is reasonable that common dolphins in these areas may have adapted to similar habitats, which may facilitate movement of individuals between these two populations.

The contrasting pattern between the nuclear and mtDNA results suggests possible female philopatry for the Alboran population. Female philopatry is not unusual in marine mammals and often population structure is more evident at the mtDNA level because of the larger dispersal of males (Hoelzel, *et al.*, 2002). However, on a worldwide scale the common dolphin does not show female philopatry (see Chapter 5). Moreover, our results did not show any significant sex-biased dispersal among the populations analysed, reinforcing the pattern observed worldwide. The analysis of the gene flow, based on a coalescent method using both

nuclear and mtDNA markers, may provide an alternative explanation. The microsatellites suggested similar gene flow among pairwise populations (Table 4). However, the mtDNA analysis suggested marked directionality of gene flow from eastern to western populations, indicating directional movements of females towards the eastern North Atlantic populations. Directional female gene flow would not imply population homogenisation but it would rather drain diversity from the 'donor' population.

The spanning network did not identify any clear lineage sorting (Fig.4). However, both the Ionian and Alboran populations were characterised by lower gene diversity and few highly represented haplotypes different from one another and not frequent among the other populations. Moreover they showed unique haplotypes, suggesting population discreteness. Two of the most common haplotypes among the Mediterranean populations are at the centre of a star-like structure, characteristic of a post-bottleneck expansion. Interestingly, all the Black Sea haplotypes are also within a star-like structure or among the commonest haplotypes, and moreover this population shows the lowest nucleotide diversity. The Atlantic populations show a different pattern with no dominance of one haplotype and significant Fu's Fs values.

We attempted to assign the samples from the central Mediterranean (and one sample from the Aegean Sea) in order to investigate the nature of the common dolphin population that underwent the recent drastic decline.

We used two different methods and the results were in some cases ambiguous. According to the probability values, some individuals were not assigned to either population, suggesting that they might come from an unsampled population. The three samples from the central Mediterranean show inconsistency in the results reinforcing this hypothesis. In fact, the samples from Tuscany, France (pre-decline) and Sicily were plotted at the border of the area defined by the reference populations in the MDS analysis, and for two of them the associated probabilities were ambiguous.

Defining population boundaries is fundamental to the formulation of effective conservation plans. Our results show a clear population boundary between the western and the eastern Mediterranean indicating the presence of discrete population in these two areas. Moreover, our results do not exclude a possible third population inhabiting the central Mediterranean Sea, as some of the samples from this area could not be clearly assigned to either population. We suggest that the population

differentiation observed in the Mediterranean Sea may have arisen from adaptation to different habitats. We suggest that the Mediterranean common dolphin population cannot be considered as a single homogeneous population and that different areas of the Mediterranean Sea should be considered independently for further actions towards the conservation of this species.

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DISCUSSION

It is clear that bottlenose and common dolphins are well adapted to a wide variety of habitats: they are found in the cool waters of Scotland and Iceland and in the tropical waters of Mexico, in both coastal and pelagic waters. Their wide distribution and adaptability is reflected in their high morphological variability. The similarity in the distribution and pattern of morphological variability suggests that these species underwent similar evolutionary processes that shaped their populations. They also show close relationship in molecular phylogenies (Le Duc *et al.*, 1999). However, in other respects, such as life history, habitat use, social structure, and feeding ecology they are different. A key difference is that common dolphins are mainly pelagic while bottlenose dolphins are mainly coastal. This thesis provides a comparative assessment of these two species, especially in the context of comparing coastal and pelagic populations of each species, towards a better understanding of the evolutionary processes leading to population structure.

Morphotypic versus genetic differentiation

Genetic differentiation was not always correlated with morphological differences in either species. For both species different morphotypes showed generally higher genetic differentiation, but not always more than populations of the same morphotype. In fact, in the bottlenose dolphin, the two populations described as *T. aduncus* based on morphology, were highly divergent and may be different species. Similarly in the common dolphin, the two populations described as *D. capensis* showed the highest pairwise genetic differentiation and were not monophyletic.

In the bottlenose dolphin, reciprocal monophyly was observed between different morphotypes (*aduncus* versus *truncatus*) and also between populations of the same morphotypes (in *truncatus*: coastal versus pelagic populations; in *aduncus* the South African versus the Chinese population). Conversely, in the common dolphin lineage sorting was not observed even between different morphotypes. The eastern North Atlantic population of *D. capensis* formed a different lineage, but the South African *D. capensis* population clustered with *D. delphis* (Chapter 4).

This is consistent with the pattern seen for some other species of small cetaceans. In Dall's porpoises (*Phocoenoides dalli*) two morphologically distinct forms (*P. d. dalli* and *P. d. truei*) showed genetic differentiation at the same level as seen for comparisons of populations of the same form. Moreover they did not show reciprocal monophyly, as twelve haplotypes were shared between the *truei* and *dalli* form, and therefore they were recognised as two forms of same species (Escorza-Trevino *et al.*, 2004).

The classification of cetacean species remains controversial. From a cladistic point of view, essentially all of the species within the *Delphinidae* are classified on the basis of superficial phenotypic similarities, and not on the basis of accepted synapomorphies (see Heyning & Lento 2002). In this context molecular studies are being used to help clarify differences among morphotypes. My data suggests that *T. aduncus* and *D. capensis* should not be considered single species on a world wide scale, as proposed by some authors (Rosel et al., 1994; Wang et al., 1999). Based on a phylogenetic approach (Chapter 2) the South African and Chinese populations of *T. aduncus* should probably be considered as two different species, while the status of a single *D. capensis* species is questionable (Chapter 4).

As stressed by some authors (Curry et al., 1997; Le Duc et al., 1999) my result reinforce the need for a more comprehensive investigation of the worldwide population genetics and morphotypic variation of these species, in order to better define their taxonomy.

Evolution of population structure

For the bottlenose dolphin, we suggested that independent founder events may have originated local coastal populations, possibly as consequence of the releasing of suitable habitats during interglacial periods, as it has been proposed for harbour porpoises (Tolley *et al.*, 2001). This was contrasted with an alternative explanation based on the differential social structure in the coastal and pelagic populations.

The comparison between common and bottlenose dolphins supports the hypothesis that habitat structure may influence population structure in these species. The bottlenose dolphin, primarily a coastal species, shows strong population structure. Conversely, the common dolphin, principally a pelagic species, shows less population structure and relatively low genetic differentiation even between different morphotypes. Moreover, in both species most of the genetic variability is partitioned

among the coastal populations. The two *aduncus* populations are coastal populations (Wang *et al.*, 1999; Cockcroft *et al.*, 1990) as are the *T. truncatus* western North Atlantic and Gulf of Mexico populations. In the common dolphin the eastern North Atlantic population of *D. capensis* is generally found in more coastal and shallower waters (Perrin, 2002).

Social structure may also be an important factor. Social structure is influenced by the feeding ecology (type and availability of prey) and ultimately by the habitat. Coastal populations of bottlenose dolphins are characterised by a complex social structure based on strong and defined relationships among the individuals of the group (fission-fusion society) and despite their capability, individuals do not generally move across long distances (Scott *et al.*, 1990; Connor *et al.*, 2000). Common dolphins on the other hand show a more fluid social structure based on aggregations of hundreds individuals (Evans, 1994).

There is evidence from the behavioural data that social structure may be a consequence of adaptation to a specific habitat. Large, fluid social groups are common for pelagic delphinid species. Striped dolphins (*Stenella coeruleoalba*), Fraser's dolphins (*Lagenodelphis hosei*) and spotted dolphins (*Stenella attenuata*) are all pelagic species that generally form large aggregations of hundreds or thousand individuals. Despite the limited information available, pelagic populations of common dolphins resemble the characteristics of other pelagic species, while common dolphins in coastal habitat seem to show social structure more like that of bottlenose dolphins (Bruno, 2001).

Pelagic habitats are more homogeneous, with environmental characteristics changing more gradually on a geographic scale. Open oceans offer few options for hiding from predators, and finding food in a disperse environment may be difficult. Therefore aggregating in big groups is likely to be advantageous, to the extent that often interspecific associations are observed among pelagic delphinids (Würsig, 2002). They often migrate following the seasonal movement of prey species.

Conversely, coastal habitats are generally heterogeneous. Physical boundaries may be present between one area and another, and topographic, oceanographic and biological features can vary consistently even across small geographic ranges. Small communities, specialised on the local food resources would be more advantageous.

Specialisation in foraging strategies would provide advantage if transferable between individuals and between generations. However transferable knowledge

implies complex social structure and long-term association between individuals (Whitehead, 1998). Gregarious, long-lived animals, such as gorillas *(Gorilla gorilla)*, deer *(Cervus elaphus)*, elephants *(Loxodonta africanus)* rely on information transfer to exploit their habitat (Conradt & Roper, 2003). Bottlenose dolphins fit in this category showing complex social structure in which the transmission of information plays an important role (Lusseau, 2003; Janik, 2000). This may explain the higher site fidelity of individuals and therefore the reduced gene flow in coastal populations.

Further evidence of a correlation between habitat structure and population structure is provided by the analysis of the population structure on a smaller geographic scale. The Mediterranean Sea represents an ideal range to test our hypothesis, as it offers a wide variety of different oceanographic environments and oceanographic discontinuities throughout the whole range.

The population structure observed in bottlenose and common dolphins in the Mediterranean Sea reflected the pattern observed on a worldwide scale: the bottlenose dolphin showed strong population differentiation, whereas the common dolphin showed less differentiation (Chapter 3 and Chapter 6). The interesting result was that for the bottlenose dolphin the population boundaries identified by the genetic analysis alone corresponded to borders between different habitat regions. The strongest boundary was identified between the Black Sea and the eastern Mediterranean Sea. Not enough samples from the Black Sea were available to test the strength of this boundary in common dolphins. However, morphological data for both species show differences in body size, life history and nursing time for both species and each Black sea population has been proposed as a different subspecies of *T. truncatus* or *D. delphis*. Moreover, it has been argued that such differences may be the result of adaptation to a specific environment (Amaha, 1994).

A second strong boundary, between the eastern and western Mediterranean, was identified in both species, despite its general lack in population structure for the common dolphin. It reflects the different preferred habitat of common dolphins in the two basins: mainly coastal in the eastern Mediterranean, and mainly pelagic in the western Mediterranean. Population differentiation between the eastern and western Mediterranean populations has also been observed in other species *(Solea vulgaris,* Guarniero *et al.,* 2002; *Dicentrarchus labrax,* Bahri-Sfar *et al.,* 2000). In these studies the authors proposed that differences in hydrographic characteristics defined the different habitats in these two areas, and promoted the differentiation of

intraspecific populations. A strong boundary was also identified for the bottlenose dolphin between Scotland and the eastern North Atlantic. This boundary was not observed in the common dolphin, as the Scottish population did not show any significant differentiation from the other eastern north Atlantic populations. This can be explained again by difference in the habitat. In Scotland the bottlenose dolphin is mainly coastal and resident populations are well documented in the area (Wilson, sometime), whereas the common dolphin occurs only seasonally and in mainly pelagic waters (Evans *et al.*, 2003).

Along the coast of KwaZulu-Natal (eastern Southern Africa), *T. aduncus* is among the most common species in the coastal waters. Observational data on the coastal populations suggested small group size, high site fidelity and restricted habitat ranges for these populations, with little movement from one area to another (Cockcroft *et al.*, 1990). Furthermore, the seasonal occurrence of a transient 'pelagic' population is documented. This population is characterised by groups of hundreds individuals following the movement along the coast of the sardine stocks in the winter season. Despite the low genetic variability that characterised this population, incipient population differentiation between the seasonal pelagic population and the resident coastal population has been observed, and may represent a further example of population differentiation correlated to habitat differences.

Implications for conservation

Understanding the population structure of a species across its habitat range and the mechanisms that lead to this population structure provides fundamental information for the formulation of conservation programmes. Conservation genetics applies genetics analysis to define units of population to conserve or Management Units (MUs). Moritz (1994) defined a MU as "... populations with significant divergence in allele frequencies at nuclear or mitochondrial loci, regardless of phylogenetic distinctiveness of alleles".

With this study I largely increase the knowledge about population structure bottlenose and common dolphins and this information can be used as a basis for further studies focusing on a more detailed assessment of their population structure to identify MUs.

For the bottlenose dolphin I identified a possible new species, the South African population defined as *T. aduncus* (Chapter 2). Moreover, I found strong

population differentiation even across small geographic ranges and, considering the possible mechanisms that may lead to this pattern (Chapter 3), further population structure cannot be excluded on a smaller local scale. Therefore, for the conservation management of this species detailed analysis of the local populations is highly recommended in order to identify which units are most important to conserve.

For the common dolphin I detected less strong population structure compared to the bottlenose dolphin (Chapter 5). For example in the eastern North Atlantic I did not find significant population differentiation suggesting that these populations may represent a single stock. However, further analysis, including samples from other areas of this region, is recommended. In fact, well-defined local populations were identified in this species (e.g. in the eastern North Pacific, in the eastern Mediterranean Sea) suggesting that despite the wide distribution, a broader study that surveys the population structure on a more local scale would be required to define units to conserve.

In this study I considered three main cases on a small geographic range that require immediate conservation effort: the Mediterranean bottlenose dolphin, the Mediterranean common dolphin and the South African bottlenose dolphin population *T. aduncus*. In these cases I managed to identify possible MUs that should be considered priorities in the formulation of conservation management strategies. A summary is provided in Table 1.

In the Mediterranean and Black Seas, threats to cetacean survival arising from human activities can be particularly severe, due to the enclosed and semi enclosed nature of such basins, and to the human density and intensity of activities, particularly in the coastal zone where habitat loss and degradation is a major concern.

Table 1. Summary of the managements units identified in this study and suggested conservation recommendations.

Population	Identified Management Units	Conservation recommendations
Mediterranean & Black Sea bottlenose dolphin (*T. truncatus*)	- Black Sea, eastern Mediterranean, western Mediterranean, eastern North Atlantic and Scottish populations	- Further investigation covering contiguous local population is recommended - High conservation measures should be taken for those populations inhabiting marginal habitats.
South African bottlenose dolphin (*T. aduncus*)	- A North coastal population - A South coastal/migratory population	- Focus on limiting the number of by-catch along the North Coast. - Promote further investigation to better assess the identity of the South coastal population versus the migratory population.
Mediterranean common dolphin (*D. delphis*)	- Western and eastern Mediterranean populations - Possible third different population in the central Mediterranean	- Higher priority for the conservation of those coastal areas where this species still occurs i.e. eastern Mediterranean and some areas in the central Mediterranean (Ischia, Malta, Sardinia). - Promote further investigation covering those areas where insufficient data are available.

In the Mediterranean and Black Seas the bottlenose dolphin shows a fragmented distribution along the coastal areas and it is likely to be heavily impacted. My results showed fine-scale population structure with boundaries that coincided with transitions between habitat regions. These results suggest that further subdivision could be found considering contiguous geographic areas. Therefore in order to define Managements Units a detailed population analysis should be conducted on a small geographic scale.

The Mediterranean common dolphin has recently undergone to a drastic decline that resulted in its disappearance from most of its historical habitat range, especially in the central Mediterranean (Bearzi *et al.*, 2003). My results provide the first insight into the population structure of this species in the Mediterranean Sea, identifying at least one population boundary: between the eastern and the western Mediterranean Sea. Moreover, they did not exclude the existence of a possible third different population inhabiting the central area of the Mediterranean Sea. Coastal habitats require local adaptation in order to permit full exploitation of their resources. However, quick changes in environmental conditions, possibly determined by anthropogenic factors, may not be followed by a fast adaptive response, therefore leading to the disappearance of populations. The common dolphin is primarily a

pelagic species, however in the eastern Mediterranean Sea it seems to have adapted to a coastal habitat, shaping its social structure. This may have happened in a period when these coastal habitats provided a favourable environment. The fact that this species is more typically pelagic may limit the rate of re-colonisation of these coastal areas and therefore facilitate the permanent disappearance of this species once the local habitat is no more suitable.

In South Africa, along the coast of KwaZulu-Natal the anthropogenic factor is the presence of shark-nets to protect bathers from shark attacks. Dolphins often get entangled and this results in a substantial by-catch, twice as high as the maximum sustainable capture rate considered for cetacean populations by the International Whaling Commission (Anon., 1991). The concern is about the impact on local populations. In this case boundaries among putative populations were identified and the populations redefined in order to estimate the actual impact on each population. This helps identify the areas that require the greatest conservation effort. The North Coast should be considered a priority and conservation plans should aim to reduce the rate of by-catches under the maximum sustainable capture rate calculated on the population estimates on this area.

In both the bottlenose and the common dolphins the most vulnerable populations are identified as those inhabiting coastal habitats and that appear to be specialised in exploiting the local resources. Coastal habitats are indeed the most heavily impacted by human activity and special effort should be concentrated on preserving those areas. However, in both species pelagic populations are also observed, but little or no data are available on these populations and their environment. A joint effort by the scientific community aiming to explore this environment is highly recommended to gain a holistic view of the populations that require the greatest conservation effort.

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The greatest reward of achieving something,
is the fact that you have achieved it.