A MOLECULAR GENETIC ASSESSMENT OF THE POPULATION STRUCTURE AND VARIATION IN TWO INSHORE DOLPHIN GENERA ON THE EAST COAST OF SOUTH AFRICA

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ABSTRACT

Coastal dolphins on the South African east coast are threatened by degradation and loss of habitat as a result of increasing coastal development, industrial effluent and agricultural runoff. In addition, dolphins off the coast of KwaZulu-Natal have, for more than four decades, been heavily exploited through unchecked incidental capture in shark nets set at 45 beaches. In light of the high rate of mortality and apparent depletion of both species, the persistence of bottlenose (*Tursiops truncatus*) and humpback (*Sousa chinensis*) dolphins in that region has been questioned.

Genetic variation in south east African dolphin populations was determined as a means of assessing the fitness of the populations and their resilience to demographic disturbances. Furthermore, in order to determine the effects of continued mortality on the KwaZulu-Natal subpopulations, it was necessary to determine whether they are open or closed to immigration from the adjacent East Cape region, which represents a relatively unstressed region, characterised by a lack of shark nets and less intensive coastal activities.

Genetic variation and differentiation in the maternal genome was assessed by determining the sequence of the first 400 bases of the mtDNA control region in bottlenose and humpback dolphins from KwaZulu-Natal and the East Cape. Nuclear variation and differentiation was estimated at six microsatellite loci and compared with earlier estimates determined from allozyme electrophoresis. Random amplified polymorphic DNA (RAPD) was assessed as a means of identifying population subdivisions and diagnostic population markers.

Both bottlenose and humpback dolphins on the South African east coast are characterised by low nuclear and organellar genetic variation, consistent with a possible genetic bottleneck, the inferred date of which coincides with the onset of the last glacial period. Genetic variation in South African bottlenose dolphins was lower than that reported elsewhere for the species, while an intraspecific comparison supported lower genetic variation in South African humpback dolphins than in humpback dolphins sampled off Hong Kong. An analysis of molecular variance (AMOVA), performed on mtDNA haplotype frequency data indicated, for both species, significant genetic subdivision, concordant with geographic location. The data suggested female bottlenose dolphins demonstrate regional philopatry, displaying limited movement between KwaZulu-Natal and the East Cape. Female humpback dolphins tend towards strict local philopatry, with significant maternal differentiation evident both within and between regional subdivisions. Differentiation in microsatellite allele frequencies was also demonstrated between KwaZulu-Natal and the East Cape for both species, suggesting that the movement of male bottlenose and humpback dolphins may also be restricted. Nonetheless, considerably higher nuclear gene flow estimates suggested that males of both species represent the principal vectors of gene dispersal.

The implications of historically low genetic variability and population subdivision in South African dolphins are important in view of the current rate of mortality in KwaZulu-Natal. The persistence of coastal dolphin populations relies on their ability to recover following a bottleneck event. Continued removal of demographically important age-sex classes such as occurs in shark nets, may not only further reduce the genetic variation, but would ultimately deplete dolphin populations in KwaZulu-Natal beyond a sustainable number, resulting in eventual local extinction. The differentiation of the two regions implies that, in the event of local extinction occurring, dolphins, particularly females, from adjacent regions will not readily re-colonise the area. This would result in fragmentation of the south east African populations and ensure reproductive isolation from neighbouring populations on the east African coast.

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ABBREVIATIONS AND SYMBOLS

bp	base pairs
BP	before the present
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
ddH$_2$0	double distilled water
EC	East Cape
EDTA	ethylenediaminetetra-acetic acid
ESU	evolutionarily significant unit
HK	Hong Kong
HWE	Hardy-Weinberg Equilibrium
IUCN	International Union for Conservation of Nature and Natural Resources
IWC	International Whaling Commission
km	kilometre
KZN	KwaZulu-Natal
m	metre
Ma	million years ago
mg	milligram
µg	microgram
mM	millimolar
µM	micromolar
MU	management unit
MY	million years
NC	north coast
Ne	effective population size
Nm	number of individuals exchanged per generation
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RAPD	random amplified polymorphic DNA
SC	south coast
SDS	sodium dodecyl sulphate
TE	TRIS-EDTA buffer
UPGMA	unweighted pairgroup method with arithmetic averaging
VNTR	variable number of tandem repeats

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Dedication

This thesis is dedicated to my parents, Denver and Dorothy Smith and my husband, Chris Goodwin, with abundant gratitude

CHAPTER 1

GENERAL INTRODUCTION

1.1 General taxonomy of bottlenose and humpback dolphins

The catholic distribution of the bottlenose dolphin (*Tursiops truncatus*) as well as its successful maintenance in captivity, makes it possibly the most well-known and well-studied of the small cetaceans. Although primarily a coastal species, *Tursiops truncatus* also occurs in large pelagic schools offshore. This eclectic use of habitat has resulted in the display of complex social behaviour patterns and diverse group dynamics. In fact, this has directed much attention to the species in an effort to define, for the purposes of conservation, global and local stock boundaries.

There is still much confusion about the taxonomic status of *Tursiops.* Several species have been assigned to the genus and many (eg. *T. gilli, T. nuuanu* and *T. gephyreus*) are still used as valid species (Leatherwood and Reeves, 1990; see also Ross and Cockcroft, 1990 for a brief review of the species), although most recognise *T. truncatus* (Montagu, 1821) as a global species. Ross (1977) recognised distinctive inshore and offshore forms on the southern African coast and assigned the two forms species status, classifying the considerably larger, offshore form as *T. truncatus* and the smaller, inshore form as *T. aduncus* (Ehrenberg). Following a review of the morphology of southern African and Australian *Tursiops*, this classification was recanted and the two forms have since been considered one species (*T. truncatus*), with the possibility of conferring subspecific status on the aduncus form (Ross and Cockcroft, 1990). However, taxonomic uncertainty has once again become the focus of studies on *Tursiops* with the presentation of recent genetic evidence for paraphyly within the genus and division of the inshore and offshore form into two species (Curry *et al.*, 1995; Curry, 1997; Le Duc, in preparation).

Inshore and offshore forms have been reported throughout most of the bottlenose dolphin's range (Shane *et al.*, 1986; Ross and Cockcroft, 1990; Mead and Potter, 1994; Wiley *et al.*, 1994), displaying distinctive habitat use, feeding and socialisation and group dynamics. The two forms have been shown to be morphologically different in both skull and external measurements (Ross and Cockcroft, 1990; Mead and Potter, 1994) and are distinguishable in some regions by their parasite load (Mead and Potter, 1994) and hematology (Duffield *et al.*, 1983) as well as by both variable and conserved regions of their mtDNA (Curry, 1997; Le Duc, in preparation).

Humpback dolphins are found in shallow tropical waters of the Atlantic, Indian and Pacific Oceans. Although five nominal species have been reported (Ross *et al.*, 1994), the Atlantic (*Sousa teuszii*, Kukenthal, 1892) and Indopacific (*Sousa chinensis*, Osbeck, 1765) humpback dolphins are the two generally recognised species (Rice, 1977). The Atlantic distribution of *Sousa teuszii* includes the coasts of Mauritania (Maigret, 1981), Senegal and the Cameroons, while the Indopacific species, *Sousa chinensis*, extends from the south eastern coast of southern Africa, across the Indian Ocean to Thailand and eastwards to the north East China Sea. Its range also extends towards the Persian Gulf and northern Red Sea in the north and Australia in the south (Ross *et al.*, 1994).

1.2 Coastal habitat and distribution of bottlenose and humpback dolphins

As a result of its proximity to human coastal activities, the inshore form of the bottlenose dolphin has been the most intensively studied. Generally smaller than the offshore form (Ross and Cockcroft, 1990), it inhabits water no deeper than 30 metres (Ross *et al.*, 1987; Wiley *et al.*, 1994) and feeds on a diversity of inshore prey, including benthic and reef prey, cephalopods (Cockcroft and Ross, 1990), mullet (Dos Santos and Lacerda, 1987; Barros and Odell, 1990; Shane, 1990) and shrimp (Barros and Odell, 1990). Inshore groups comprise, on average, 1-100 dolphins (Shane *et al.*, 1986), although larger schools have been recorded on the southern African coast (Saayman and Tayler, 1973; Peddemors, 1995). In many areas, schools displaying varying degrees of cohesiveness (Shane *et al.*, 1986; Scott *et al.*, 1990; Bräger *et al.*, 1994) can be found within definable home ranges (Shane *et al.*, 1986; Ross *et al.*, 1987; Scott *et al.*, 1990; Würsig and Harris, 1990). However, significant site fidelity appears to be restricted to shallow, protected bays and channels (Scott *et al.*, 1990), while unprotected, open coastline habitats appear to support more dynamic group affiliations (Ballance, 1990, 1992). Moreover, ranges have been observed to change seasonally, in response to prey movements (Shane *et al.*, 1986; Scott *et al.*, 1990), with a tendency towards migrations or range extensions (Kenney, 1990; Wells *et al.*, 1990).

Strictly an inshore species, the humpback dolphin prefers a shallow coastal habitat (Corkeron, 1990) and occurs around estuaries (Cockcroft, 1991; Zhou *et al.*, 1980), inshore reefs (Saayman and Tayler, 1979) and delta regions (Zbinden *et al.*, 1977). This is reflected in the prey taken by the species on the east coast of southern Africa, which includes inshore, demersal fish and cephalopods (Saayman and Tayler, 1979; Barros and Cockcroft, 1991). This feeding strategy appears to influence strongly the size of humpback dolphin schools. Group sizes of fewer than 10 individuals are commonly reported (Saayman and Tayler, 1973; Zbinden *et al.*, 1977; Saayman and Tayler, 1979; Ross, 1984) with individuals dispersing to feed individually or in small groups (Saayman and Tayler, 1979). Unlike the structure observed in bottlenose dolphins, humpback dolphin groups do not appear to be segregated into age or sex classes (Saayman and Tayler, op cit). The year-round observation of the species in Plettenberg Bay (Saayman and Tayler, op cit.) and Richards Bay (Durham, 1995) and the continual resighting of identifiable individuals, implies the species may observe a high degree of local residency.

1.3 Conservation and management of inshore cetacean populations

1.3.1 Threats to coastal cetacean populations

As top predators and in some cases, prey, cetaceans are intrinsically interwoven into the dynamics of the marine ecosystem. Furthermore, since the realisation of the economic viability of regulated whale-watching and the subsequent 1986 imposition of a moratorium on whaling by the International Whaling Commission (IWC; Donovan, 1991), many countries are developing lucrative tourist markets around the occurrence of large and small cetaceans (Morris, 1988).

Despite the virtual cessation of commercial whaling, cetaceans continue to be affected by both incidental and directed exploitation. The coastal distribution of many cetacean species exposes them to directed take (Cooke, 1991), incidental take (Cockcroft and Krohn, 1994; Lal Mohan, 1994), pollution, habitat destruction and fragmentation, depletion of food sources and disturbance (Cooke, 1991). The strictly coastal humpback dolphin represents just one species believed to be suffering a decline in many areas as a result of one or all of these factors (Pilleri and Pilleri, 1979; Cockcroft, 1990a,b; Cockcroft and Ross, 1991; Lal Mohan, 1985, 1994).

Although directed take may account for a greater proportion of dolphin mortality than estimated, incidental by-catch in gill net fisheries is perhaps a larger problem for coastal dolphins in developing countries (Lal Mohan, 1985, 1994; Cockcroft, 1992; Cockcroft and Krohn, 1994), where intensive commercial and subsistence fishing occurs in coastal waters. Humpback dolphins have been reported commonly in fisheries by-catch for areas such as West Africa (Maigret, 1981), the Indus Delta (Pilleri and Pilleri, 1979) and the southwest coast of India (Mohan, 1985, 1994). Interaction with commercial fisheries occurs in almost all of the bottlenose dolphin's range (Northridge, 1991). Between 1976 and 1980, bottlenose dolphins comprised 32% of the total by-catch in gill nets set off the Calicut coast, India (Lal Mohan, 1985). Furthermore, the introduction of synthetic gillnets and driftnets has increased dolphin mortality along the Indian coast to more than 1000 animals annually, of which bottlenose and humpback dolphins are two of the most prevalent species captured (Lal Mohan, 1994). Although no accurate data are available, there have been reports of incidental entanglement of bottlenose dolphins in fishing gear in Madagascar and Mozambique (Cockcroft and Krohn, 1994), while unchecked exploitation in the form of incidental capture in shark nets has occurred since the 1950s off the east coast of South Africa (Cockcroft, 1990b).

Unmanaged coastal development and agriculture results in the destruction of fish nurseries, thus reducing the food supply for higher predators, while pollutants enter the food chain and accumulate in the tissues of both predators and prey, potentially resulting in mortality and reduced reproductive efficiency (Subramanian *et al.*, 1987; Cockcroft *et al.*, 1989b; 1990a, 1991). Die-off events as a possible result of environmental contaminants and/or pathogenic diseases have reduced coastal bottlenose dolphin numbers along the Atlantic coast and Gulf of Mexico (Curry and Smith, 1997), while several species of coastal marine mammals off South Africa have been found to accumulate toxic organochlorine residues in their blubber (Cockcroft *et al.*, 1989b; 1990a; 1991a).

Bottlenose and humpback dolphins are listed as category K species in the IUCN Red Data book (Cooke, 1991; Groombridge, 1993). This implies that the species are suspected to be threatened, vulnerable or endangered, but cannot be conclusively placed in any of these categories due to a lack of information. However, they are known to be taken, either directly or indirectly throughout their inshore range, while their habitat is vulnerable to encroachment, disturbance and pollution (Cooke, 1991). While bottlenose dolphins are regarded as Appendix II species, CITES lists humpback dolphins in Appendix I, as a result of the vulnerability of their inshore habitat and because directed takes for human consumption may still occur in the Arabian Sea, Red Sea and Persian Gulf (Cooke, 1991).

In many coastal regions, particularly in developing countries, population numbers of inshore dolphins are declining due to excessive exploitation and the lack of effective means of conservation (Obura *et al.*, 1996). The consequences of failure to curb the decline of diminishing populations are well-documented (Lande, 1988; Amos and Hoelzel, 1992). In the case of marine mammals, this is particularly significant for threatened or endangered species (Patenaude *et al.*, 1994) and those recently or currently exploited (Cooke, 1991; Daníelsdóttir *et al.*, 1991; Hoelzel and Dover, 1991b; Wada and Numachi, 1991; Baker *et al.*, 1993, 1994).

1.3.2 How can cetacean populations be conserved or managed ?

The primary objective of applied conservation biology is to avert extinctions (Goodman, 1987). Motivation for conservation has traditionally been based on ecological, economic or even ethical principles (Franklin, 1980). Recently, however, more emphasis has been placed on investigation of the genetic component of population structure. It is becoming clear that both genetic and demographic factors feature prominently among the many causes of local extinction and many case studies have revealed the failure of conservation initiatives on the basis of insufficient consideration of either genetic or demographic issues (Lande, 1988; Avise 1989; Avise and Nelson, 1989).

The need to determine the discreteness of conspecific populations in order to define the boundaries of populations requiring conservation is foremost in any conservation initiative. This may be achieved with a combination of demographic and genetic studies. Until recently, the determination of stock structure and population demographics of cetaceans was conducted by field and aerial surveys (eg. Dos Santos and Lacerda, 1987; Cockcroft *et al.*, 1990b, 1991b), observations from purse-seiners and whaling ships (eg. Perrin *et al.*, 1982, 1983) and tagging (eg. Perrin, 1975; Perrin *et al.*, 1979). However, where the demographic study of cetacean species is hindered, genetic studies may prove to be the only economical means of determining the structure and dynamics of a population.

Managers are becoming increasingly aware of the need to determine the genetic structure and variability of populations and species (Soulé and Wilcox, 1980; Soulé, 1986) in order to best define management units (Lande, 1991). Studies of cetaceans may be indicative that the maintenance of genetic diversity is imperative in natural populations, particularly where population numbers are low or habitat is fragmented or reduced (Schaeff *et al.*, 1991; Patenaude *et al.*, 1994).

1.3.3 The use of genetic studies in the conservation and management of cetacean populations.

The role of genetics in the conservation of natural populations is to propose minimum demographic and genetic criteria which would prevent the effects of genetic drift and increase resilience to stochasticity (Soulé, 1986). Genetic analyses are likely to be most informative when interpreted within a demographic, behavioural or comparative framework (Moritz, 1994) and the combined use of genetic methods and field and photographic data has provided a wealth of information about the life history and breeding behaviour of social cetacean species.

Earlier genetic studies using large samples acquired from traditional harvests (Andersen, 1988) and commercial whaling forays (Shimura and Numachi, 1987; Wada, 1988; Daníelsdóttir *et al.*, 1991; Wada and Numachi, 1991) investigated population structure and genetic variation in allozyme systems. However, studies of population structure require sufficient levels of intraspecific variability (Murphy *et al.*, 1991) which is often not available in the allozymes of some organisms, making DNA techniques more applicable. Practical considerations have become an important determinant in the choice of molecular analysis used in population genetic studies. One of the most important shortcomings of allozyme analysis may be the deterioration of the enzyme activity required for the visualisation of allozymes, due to length and conditions of storage. Furthermore, seasonal (Amos and Hoelzel, 1992) or tissue-specific (Murphy *et al.*, 1991) expression of allozymes may reduce the reliability of the system.

Recent advances in molecular DNA technology have fortified population genetics studies with techniques which can be applied ubiquitously to plant and animal tissues. The use of DNA markers has several advantages over protein and allozyme studies. Free from tissue and seasonal variation, DNA is relatively stable; however, its utility lies in the ease with which several different types of analysis can be conducted. Use of appropriate techniques, rendering sufficient maternally-, paternally- and biparentally-inherited marker loci should make possible accurate estimations of population structure, dispersal and gene flow, phylogenetic relationships, degrees of relatedness and effective population size (Baker, 1994).

Mitochondrial DNA (mtDNA) may be particularly useful for studies of cetacean populations because the social organisation of schools and/or populations is, in many cases matriarchal, with male dispersal accounting for almost all of the genetic exchange (Duffield and Wells, 1991; Amos *et al.*,1993; see section 2.1.1 for a brief review of the use of mtDNA in population genetics studies). The utility of mtDNA in conservation genetics lies in the measure of genetic variation within populations, particularly those experiencing a decline in numbers. It may also be used to identify populations or regions which are evolutionarily divergent or to determine the phylogenetic validity of previously-identified stocks or populations (Baker *et al.*, 1994; Moritz, 1994).

Analysis of restriction fragment polymorphisms (RFLP) has provided insight into the genetic structure of geographic forms of the incidentally-killed spinner dolphin (*Stenella longirostris*) in the Eastern Tropical Pacific (Dizon *et al.*, 1991) and Dall's porpoise (*Phocoenoides dalli*) in the western North Pacific and Bering Straits (McMillan and Bermingham, 1996). Using mtDNA RFLP analysis, Dowling and Brown (1993) determined that populations of bottlenose dolphins from the US Atlantic coast were genetically differentiated from those in the Gulf of Mexico, while Baker *et al.* (1990) determined that the genetic differentiation of the maternal genome of humpback whales was a consequence of migratory fidelity.

The ability to amplify DNA by means of the polymerase chain reaction (PCR, Mullis and Faloona, 1987; Saiki *et al.*, 1988) has revolutionised the study of previously intractable cetacean populations (see section 2.1.2). Whereas early sampling efforts focused on commercial harvests, strandings and incidental net captures (Andersen 1988, Amos *et al.*, 1991; Dizon *et al.*, 1991), current population studies make use of the biopsy method (Mathews *et al.*, 1988; Whitehead *et al.*, 1990; Palsbøll *et al.*, 1991) or the retrieval of sloughed skin (Amos *et al.*, 1992) to acquire samples from cetaceans. These methods enable the relatively non-invasive sampling (Lambertsen, 1987; Brown *et al.*, 1991; Weinrich *et al.*, 1991) of free-ranging cetaceans and will permit collection from specific individuals for group or kinship analysis. Furthermore, DNA retrieved from stranded or incidentally-captured carcasses may be amplified sufficiently for population analysis (this study). Direct sequencing of specified regions of DNA has been expedited and simplified by prior amplification of the target DNA. Using sequence information from the mtDNA control region and cytochrome b gene, Rosel *et al.* (1994) confirmed the reproductive isolation of two sympatric forms of the common dolphin (*Delphinus delphis*) in the Northeast Pacific. Furthermore, historical population structure has been inferred from mtDNA sequence data for humpback whales (Baker *et al.*, 1993, 1994) and killer whales (Hoelzel and Dover, 1991a), while a contentious debate about evolutionary relationships within the cetacea (Douzery, 1993; Milinkovitch *et al.*, 1993; Arnason and Gullberg, 1996) has made use of sequence information of the more conserved genes of the mtDNA genome.

The use of multilocus probes which target tandem repeats in the genome, generating DNA 'fingerprints' (Jeffreys *et al.*, 1985a, b), has provided novel information about the genetic and social structure of cetacean schools (Amos and Dover, 1990). Multilocus DNA fingerprinting was used successfully to test for paternity and kinship in pilot whale pods (Amos *et al.*, 1991) and analysis of hypervariable regions has been useful in assigning paternity in killer whales in situations where it was indeterminable from field observations (Hoelzel *et al.*, 1991a). Patenaude *et al.* (1994) suggested from band-sharing data obtained by DNA fingerprinting that beluga whales in the St. Lawrence Estuary may be suffering inbreeding depression, leading to a loss in genetic fitness.

Amplification of hypervariable regions (Litt and Luty, 1989; Tautz, 1989) and random genomic loci (Welsh and McClelland, 1990; Williams *et al.*, 1990) by means of the PCR, have rapidly become widely-used methods of classifying and identifying individuals and populations based on genotype frequency data. Allele frequency data from variation at six microsatellite loci (see section 2.1.3) supported a matrifocal organisation of pilot whale pods (Amos *et al.*, 1993), while a similar study on pinnipeds (Allen *et al.*, 1996) demonstrated a degree of regional female philopatry in grey seals.

The use of genetic methods to resolve population questions raised by morphological and demographic data is expanding as the resolution of molecular techniques improves. With the reciprocal use of molecular and demographic research methods in marine mammal science, hitherto unstudied and currently endangered populations and species are more likely to benefit from concerted conservation efforts and more effective management programs.

1.4 South African inshore dolphin populations

1.4.1 Conservation of inshore dolphins off the east coast of South Africa

Both species appear to occur in nearshore regions along the entire east coast of South Africa (Ross, 1984). However, while coastal bottlenose dolphins have been observed to move further offshore than they ordinarily occur (Ross *et al.*, 1987), humpback dolphins appear to prefer a strictly nearshore distribution (Durham, 1995; Karczmarski, 1996). This proximity to the shore exposes both species to numerous hazards, both natural and anthropogenic, with the result that populations in certain regions are starting to show signs of depletion (Cockcroft *et al.*, 1991b, 1992; Durham, 1995).

Agricultural activities along the length of the coast have contributed to the siltation of rivers and consequently, disruption of the ecology of many estuaries

(Cockcroft, 1990a). This has a direct and immediate effect on humpback dolphin populations which tend to congregate around the major estuary systems (Durham, 1995; Karczmarski, 1996) in favour of the prey species which breed there. In particular, mangrove swamps, which are prevalent on the KwaZulu-Natal (KZN) north coast (Fig. 2.2) provide shelter for adult fish and nursery areas for the fry of many of the humpback dolphin's prey species. However, the same area is subject to coastal development, dune mining and is the country's primary sugar-growing region. Coupled with the siltation and pollution of rivers and estuaries, the result of this industrial and agricultural activity is the leaching of organochlorine contaminants such as polychlorinated biphenyls (PCBs), DDT and dieldrin (Cockcroft *et al.*, 1989b, 1990a) into the rivers and ocean. Although the use of dieldrin was apparently terminated in 1982, significant levels of the toxin have been found to accumulate in the blubber of marine mammals and seals (Cockcroft *et al.*, 1991a). Furthermore, DDT, used extensively in agriculture and in the control of malaria, may still be entering the ecosystem (op cit.).

Residues of all three contaminants have been found to accumulate in marine mammals at levels which may contribute to mortality and loss of fecundity (Cockcroft *et al.*, 1991a). Bottlenose and humpback dolphins inhabiting inshore waters off both the KZN and East Cape coasts, displayed higher levels of accumulation than those found further offshore (Cockcroft *et al.*, op cit.). Moreover, a decline in the amount of contamination in sexually mature female bottlenose dolphins suggested that a significant amount is offloaded to the first calf during lactation (Cockcroft *et al.*, 1989b). Although the effects of this are unknown, high levels of contamination may contribute significantly to neonatal mortality. Furthermore, male bottlenose and humpback dolphins display levels of contamination in excess of those implicated in reduced testosterone production elsewhere (Subramanian *et al.*, 1987; Cockcroft *et al.*, op cit.).

A high rate of natural mortality due to predation is suspected in coastal cetaceans off South Africa. Estimates made from the number of sharks found to contain bottlenose dolphin remains at the time of capture and the number of bottlenose dolphins showing signs of previous attack, suggest at least 20 bottlenose dolphins are killed each year by shark predation (Cockcroft *et al.*, 1989a). The humpback dolphin's tendency towards turbid estuarine waters increases the incidence of attack on this species by large sharks. Cockcroft (1991) reported a minimum of 28% of the humpback dolphins caught incidentally in shark nets over a seven year period, showed signs of previous attack by sharks.

The exploitation of coastal dolphins along the KZN coast commenced earlier this century, when dolphin populations were so large that the Natal Provincial Government offered a £5 bounty for every dolphin killed, in order to protect fish stocks (Cockcroft, personal communication). In 1952, shark nets were installed at Durban (Fig. 2.1) in an effort to reduce the incidence of bather attack by large sharks. There are currently over 400 nets set at 45 bathing beaches along the KZN coast (Cockcroft, 1990b; Cockcroft and Ross, 1991; Peddemors *et al.*, 1991; Peddemors, 1995).

Incidental capture in shark nets presents the greatest hazard to inshore dolphins along the KZN coast. Cockcroft (1990b) reported an incidental bycatch of 279 bottlenose dolphins over a nine year period, no less than 25% of which comprised lactating females and calves (Cockcroft, 1992). Durham (1995) reported the capture of 95 humpback dolphins over a 12 year period, 74% of which were taken from the four most northern netting installations. This rate of mortality far exceeds the 2% considered by the IWC as the maximum sustainable capture rate for a dolphin population (Anon, 1991). In fact, assuming an annual natural replenishment rate of 3-5% (sensu Reilly and Barlow, 1984), the average annual bycatch of 3-4% and 3-5%, respectively, of the estimated bottlenose and humpback dolphin populations (Cockcroft, 1990b, 1992; see section 1.3.3 below), may be depleting the KZN populations beyond a sustainable rate.

The South African east coast represents the edge of the Indo-Pacific range for both species, increasing the potential for reproductive isolation of local populations. This situation is exacerbated by the increasing pressure faced by coastal dolphins along the rest of the east coast of Africa and the motive to conserve locally is augmented by the need to conserve bottlenose and humpback dolphins at a global level. A workshop addressing the conservation of the Indian Ocean, held in Mombasa, Kenya, 1995 (Obura *et al.*, 1996) lists the humpback dolphin as one of the most critically threatened coastal mammals in the Indian Ocean, citing incidental captures, habitat loss and degradation, disturbance and local extinction as the most important issues facing the conservation of the species. The report recommends genetic analysis of all populations and species of *Sousa* as one of the research methods requiring top priority in the effort to conserve the genus.

The IUCN/SSC Action Plan (Perrin, 1989) proposes an assessment of the effects of incidental takes of humpback dolphins in shark nets off the coast of KZN, South Africa, while at a co-hosted meeting on the 'Mortality of Cetaceans in Passive Fishing Nets and Traps' (IWC, 1990), it was concluded that bottlenose and humpback dolphins along the eastern coast of South Africa were among those species which will be unable to sustain current levels of removal through incidental takes of gill net fisheries.

Much work has already been conducted on aspects of their biology which may assist in the conservation of inshore dolphins in South Africa (see below). However, additional information on the population dynamics and structure of both species is required in order to assess the impact of incidental captures on their persistence off the KZN coast and the effect of depletion on schools or populations on the remainder of the coast.

1.4.2 Current knowledge of the dynamics of inshore dolphins off the east coast of South Africa.

The past two decades have seen the collection of a considerable amount of information on southern African inshore dolphin species by a mere handful of researchers. Ross (1984) characterised the composition of small cetacean fauna found off the east coast, identifying those species which are most commonly observed. By virtue of their coastal distribution, bottlenose and humpback dolphins have required and attracted the most research effort. Early field studies conducted in the eastern Cape, provided preliminary information on the group sizes and dynamics of both genera (eg. Saayman and Tayler, 1973), while captive studies provided additional information on the behaviour of bottlenose dolphins (Saayman *et al.*, 1973). The first comprehensive study of the behavioural ecology of the humpback dolphin was conducted between 1970 and 1973 (Saayman and Tayler, 1979) and is still regarded as a benchmark in the study of this elusive and esoteric species. Subsequent studies on the demography and group dynamics of both species have provided substantial insight into the biology, census and structure of South African populations (Durham, 1995; Peddemors, 1995, Karczmarski, 1996).

Population estimates have been made for both species from aerial surveys and photo-identification studies. Between 250 (Ross *et al.*, 1989) and 350 (Cockcroft *et al.*, 1991b) bottlenose dolphins have been estimated to occur off the KZN south coast, although Peddemors (1995) has estimated in excess of 700 animals during peak abundance in the winter months. Estimates of 430 (Ross *et al.*, 1989), 520 (Cockcroft *et al.*, 1992) and 600 (Peddemors, 1995) bottlenose dolphins have been reported for the north coast, suggesting a total population of 600 - 1000 individuals on the KZN coast. Although an accurate estimate of bottlenose dolphins off the East Cape coast is not available, Karczmarski (unpublished data) has photographically identified over 1000 individuals in Algoa Bay and St. Francis Bay (Figs. 2.1 and 2.2).

The number of humpback dolphins occurring off the KZN coast has been estimated at between 160 and 200 animals (Ross, 1982; Durham, 1995). Although accurate estimates are unavailable for the remainder of the south east coast, Karczmarski (1996) proposed the ocurrence of 200-400 humpback dolphins in Algoa Bay and the adjacent south western coastal regions alone (Fig. 2.2). When the population estimates for the KZN and East Cape coasts are compared for both species, the impact of increased pressure in KZN becomes clearer.

1.4.3 Objectives of this study

The persistence of inshore dolphins off the KwaZulu-Natal coast can only be ensured by the curtailment of exploitation pressure. Nonetheless, it is imperative that the impact of continued mortality in shark nets and exposure to toxic pollutants is assessed in order to formulate a strategy for conservation. Continued removal of demographically important individuals from a small population would reduce the average fitness of individuals and ultimately, the viability of the population (Allendorf and Leary, 1986; Amos and Hoelzel, 1992). In order to assess the resilience of both species to stochasticity, knowledge of the existing level of variation is required. Moreover, the continuity or differentiation among schools or groups in areas heavily affected by depletion pressure and those exposed to fewer hazards, must be assessed so that a strategy for conservation can be assessed in light of current exploitation and measures can be implemented to either curtail or offset mortality.

This study was initiated as a result of growing concern that the numbers of both species off the KZN coast were declining due to sustained capture in shark nets (Crockcroft and Ross, 1991). It aims at evaluating the genetic variation in bottlenose and humpback dolphins exposed to incidental capture in order to assess the effects of persistent depletion on apparently resident schools (Ross *et al.*, 1987; Cockcroft *et al.*, 1990b). The annual influx of bottlenose dolphins into KZN from the southern regions, most notably from the unnetted and therefore, less stressed East Cape region is purported to offset the shark net mortality of that species in KZN (Peddemors, 1995). However, data on the structure of South African dolphin populations are cursory and a preliminary genetic study implied that bottlenose dolphins off KZN and the East Cape may comprise separate stocks (Goodwin *et al.*, 1996).

Localities along the KZN coast which are characterised by high sighting frequency are suspected to represent 'preferred areas' (Ross *et al.*, 1987; Cockcroft *et al.*, 1990b), analogous to the defined home ranges observed for coastal bottlenose dolphins elsewhere (Shane *et al.*, 1986; Scott *et al.*, 1990; Wells and Scott, 1990). The degree of fidelity of dolphins to putative home ranges requires investigation so that the effect of mortality at heavily netted localities may be assessed. Available field data are unable to distinguish dolphins from KZN or the East Cape, nor will they permit inferences to be made about the discreteness or homogeneity of putative home ranges. Thus, in addition to assessing the genetic variation in KZN dolphins, this study aims to determine, from genetic data whether fidelity to 'preferred areas' within KZN is observed and whether KZN and East Cape dolphins constitute discrete stocks.

Previous field studies conducted off South Africa have intimated that humpback dolphins display a high degree of residency in certain areas (Saayman and Tayler, 1979; Durham, 1995). However, knowledge of the dynamics of the species along the coast and the interaction of animals from different localities is unknown. This study aims at assessing both the genetic variability of humpback dolphins exposed to shark net capture and the fidelity of the species to defined regions or localities in order to determine the effect of sustained capture off the KZN coast.

Sequence variation in the first 400 bases of the mitochondrial control region is used to assess the maternal evolutionary structure and diversity of both genera from the east coast of South Africa. Allele frequency variation in hypervariable microsatellite loci is investigated to determine the level of population structure and differentiation with respect to nuclear DNA markers. Finally, the use of RAPD markers is critically evaluated as an expedient and diagnostic means of identifying populations.

The findings of this study are integral to the proposal of a strategy for conserving inshore dolphins off South Africa. However, the data are best considered in the context of available demographic evidence. Thus, the results of the study will be discussed in light of current information on the two genera and more specifically, the populations in question in order to present appropriate recommendations for their management and sustainability.

Chapter 2 presents an account of the study area and laboratory methodology and includes a review of the molecular techniques employed in the investigation. Part I investigates bottlenose dolphins and includes Chapter 3, which describes the genetic diversity in the molecular systems studied, Chapter 4, which estimates the mtDNA phylogeny and evolutionary history of the species in South Africa and

Chapter 5, which investigates the structure and gene flow within and between KZN and the East Cape. Part II investigates humpback dolphins and comprises Chapter 6, in which the genetic diversity, mtDNA phylogeny and evolutionary history are discussed and Chapter 7, which determines the structure and gene flow within the South African population. Since this study represents the first genetic investigation of humpback dolphins, no published data exist by which intraspecific, interpopulation comparisons can be made. Thus individuals sampled off the coast of Hong Kong are included in this study as a means of comparing the genetic structure and variability of the South African population with that of a vicariant, conspecific population. The final chapter concludes the study and in consideration of conservation theory, provides recommendations for the management of the populations concerned.

CHAPTER 2

STUDY AREAS AND LABORATORY METHODOLOGIES

2.1 INTRODUCTION

2.1.1 Mitochondrial DNA analysis

Mitochondrial DNA has proved to be useful in defining relationships among closely-related species and has become an efficient means of differentiating conspecific populations. Animal mtDNA is a duplex, closed circular molecule ranging in size from 14 to 30 kilobase (kb) pairs, with an average size of approximately 16.4 kb common in mammals (Moritz *et al.*, 1987). Comprised primarily of coding sequences, animal mtDNA displays high levels of conservation in gene order and content, with the major coding regions including two rRNAs, 22 tRNAs and a further 13 genes coding for proteins involved in electron transfer and ATP synthesis (Moritz *et al.*, 1987). Nonetheless, there remains a high level of detectable heterogeneity in mtDNA sequences, both within and among populations (Avise *et al.*, 1979), making it a useful locus for population studies.

Several characteristics unique to mtDNA offer important advantages over nuclear DNA markers. A maternally-inherited and thus effectively haploid genome, mtDNA is not influenced by recombination (Avise *et al.*, 1979) and is, therefore, an effective means of assessing population demographics and female philopatry. An important consequence of maternal inheritance is the reduction in effective population size to one quarter that of nuclear genes (Birky *et al.*, 1983), resulting in a higher rate of differentiation by random genetic drift. Furthermore, stochastic processes become important factors influencing the frequencies of mtDNA haplotypes, making it an ideal marker for tracing recent evolutionary history, including founder events and bottlenecks (Harrison, 1989).

Although recent studies have suggested that the rate of evolution may be slower in cetaceans (Hoelzel *et al.*, 1991b, Hoelzel and Dover, 1991b; Baker *et al.*, 1993), studies of mtDNA evolution suggest an average rate of divergence of 2% per million years for most mammals, 5-10 times the rate observed for single copy nuclear DNA (Brown *et al.*, 1979; Wilson *et al.*, 1985). This may, to a large extent, be influenced by the especially rapid evolution of the noncoding control region. This 900 bp stretch includes the origin of mtDNA heavy strand replication and the displacement loop (D-loop) and has been shown to evolve up to five times faster than other regions of the genome (Aquadro and Greenberg, 1983). This rapid rate of evolution and the generally homoplasmic (Avise *et al.*, 1979, but see Bentzen *et al.*, 1988, Densmore *et al.*, 1985 and Hoelzel *et al.*, 1994 for exceptions) nature of the mitochondrial genome makes it especially useful in the study of population subdivision and intraspecific phylogeographic variation.

Specific isolation of organellar DNA and subsequent digestion with restriction endonucleases (Brown, 1980; Lansman *et al.*, 1981) generates restriction fragment length polymorphisms (RFLP), the variation between which may be used in the determination of population structure (Avise *et al.*, 1979; Avise *et al.*, 1992), phylogeny (Avise and Nelson, 1989; Brown and Simpson, 1981; De Salle and Giddings, 1986), phylogeography (Ball *et al.*, 1988; Ellsworth *et al.*, 1994) and genome mapping (Ferris *et al.*, 1983; Cann *et al.*, 1987; Ohland *et al.*, 1995). The same information can be achieved from a total DNA extract, from which RFLPs are detected with radiolabelled oligonucleotide probes (Graves *et al.*, 1984; Garcia and Davis, 1994). The analysis of RFLP markers has been used to characterise the maternal genome in a variety of animals, from insects (Sperling and Harrison, 1994), amphibians (Lamb and Avise, 1986), reptiles (Bowen *et al.*, 1989) and fish (Gold and Richardson, 1991; Graves and Dizon, 1989; Graves *et al.*, 1984; Hynes *et al.*, 1989; Mulligan and Chapman, 1989; Quattro *et al.*, 1990) through birds (Ball *et al.*, 1988; Zink, 1994), terrestrial (Ellsworth et al., 1994; Ferris *et al.*, 1981; Wayne *et al.*, 1991) and marine mammals (Dowling and

Brown, 1993; McMillan and Bermingham, 1996; Ohland *et al.*, 1995) and humans (Brown, 1980; Cann and Wilson, 1983; Cann *et al.*, 1987; Denaro *et al.*, 1981).

The innovation (Maxam and Gilbert, 1977; Sanger *et al.*, 1977) and recent automation (Rosenthal and Charnock-Jones, 1993; Halloran *et al.*, 1993) of nucleotide sequencing has enhanced the resolution of DNA studies to the point that single base changes can be detected. Cloning and amplification of specified regions of a genome and subsequent sequence determination has enabled the estimation of nucleotide variation and rates of evolution of individual genes (Douzery, 1993; Irwin *et al.*, 1991; Hoelzel *et al.*, 1991b). Nucleotide diversity and sequence variation can, in addition, be used to confirm population structure and phylogeny suggested by demographic methods and morphology (Baker *et al.*, 1994; Rosel *et al.*, 1994) or to determine such where less sensitive molecular and demographic methods were inconclusive (Fajen and Breden, 1992; R.J. Baker *et al.*, 1994).

2.1.2 Polymerase chain reaction (PCR)

Earlier studies requiring large amounts of a particular fragment of DNA made use of laborious bacterial cloning methods to amplify the target region prior to sequencing of the fragment (Higuchi *et al.*, 1984; Pääbo 1985). The procedure is suitable for once-off studies and for characterisation of whole genomes (Gemmel *et al.*, 1994), but is impractical for the analysis of several samples. Initially these methods were successful in amplifying sufficient ancient DNA to be sequenced (Higuchi *et al.*, 1984; Pääbo 1985), but problems such as low cloning efficiency and the occurrence of cloning artifacts hindered progress and the reliability of results (Pääbo *et al.*, 1989; Pääbo and Wilson, 1988).

The polymerase chain reaction (Mullis and Faloona, 1987; Saiki *et al.*, 1988) is an in *vitro* method of enzymic synthesis of a target DNA fragment. Defined by single-stranded oligonucleotide primers the desired fragment is replicated a million-fold or more by a thermostable DNA polymerase, during repeated cycles of template denaturation, primer annealing and target extension. The result is an exponential amplification of the target fragment which can be sequenced, probed or digested with restriction enzymes. The procedure will not be dealt with in detail here, but is comprehensively reviewed in Arnheim *et al.* (1990) and Ehrlich (1989).

The PCR has become a routine component of diagnostic, forensic and research protocols (Kirby, 1990; Goodwin and Meintjes-van der Walt, 1997). Additionally, population genetics studies make use of the efficiency with which the procedure can isolate and amplify tandemly-repeated simple sequences (Amos *et al.*, 1993; Ellegren, 1991). However, the power of PCR is best realised when the product is used to determine the sequence of a gene or comparable region of DNA. This has vastly increased the resolution of phylogenetic reconstruction, particularly where museum collections allow the study of relationships between modern populations and their ancestral and extinct congeners (Pääbo and Wilson, 1988; Thomas *et al.*, 1989).

Use of the PCR, together with conventional and automated DNA sequencing in population genetics studies, has facilitated the analysis of gene sequences of large numbers of samples. Furthermore, the flexibility of annealing temperatures and directional bias of the polymerase enzyme has enabled hybridisation of primers with sequences of limited homology, such that conserved primers can be used to amplify the DNA of species lacking in sequence information (Kocher *et al.*, 1989). Finally, the PCR presents a distinct advantage in the study of endangered or inaccessible species. Amplification of mtDNA isolated from cetacean skin, provides an effective means of studying population structure in these species. Small skin biopsies are now routinely used as an amplifiable DNA source from free-ranging dolphins and whales (Palsbøll *et al.*, 1992; Baker *et al.*, 1993), while the retrieval of sloughed skin from whales provides a non-intrusive means of assessing pod and individual relationships (Amos *et al.*, 1992). The power of the PCR was demonstrated particularly well when mtDNA amplified from faeces of a population of endangered European brown bears showed that not only could bear mtDNA be amplified, but the chloroplast *rbc*L gene could be detected, sequenced and identified, enabling an investigation of the feeding behaviour of the bears during the sampling season (Hoss *et al.*, 1992).

2.1.3 Microsatellite markers

Hypervariable microsatellite DNA sequences comprising di-, tri- or tetranucleotide tandem repeats are distributed widely throughout the eukaryotic genome (Tautz *et al.*, 1986; Edwards *et al.*, 1992). Independently characterised, the analysis of microsatellite loci (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989) exploits the power of PCR amplification to detect length polymorphisms believed to be generated by polymerase slippage during replication (Tautz, 1989; Schlötterer and Tautz, 1993). The extensive polymorphism within these loci results in the characterisation of several highly informative alleles, making microsatellite DNA an excellent source of testable molecular variability. Loci are selectively neutral, displaying Mendelian inheritance and codominance, making them ideal markers for investigating intra- and inter-population diversity, genome mapping and kinship studies (Tautz, 1989).

Although gaining optimal benefit from microsatellite analysis requires the isolation and characterisation of species-specific loci (Hughes and Queller, 1993; Scribner *et al.*, 1994; Taylor *et al.*, 1994), an unusually high degree of conservation exists in the sequences flanking the repeats, with the result that primers designed for one species will frequently resolve microsatellite polymorphisms for closely-related taxa (Schlötterer *et al.*, 1991; Gottelli *et al.*, 1994, Moore *et al.*, 1991; Pépin *et al.*, 1995). Careful selection of primers will allow the resolution of several loci simultaneously on a sequencing gel. Furthermore, recent advances have promoted automation of the analytical procedure (Ziegle *et al.*, 1992). Together, these factors provide an efficient and relatively inexpensive means of screening several highly informative DNA markers.

The utility of microsatellites has been demonstrated in population studies of animals as diverse as humans (Edwards *et al.*, 1992) cetaceans (Amos *et al.*, 1993), marsupials (Taylor *et al.*, 1994), canids (Gottelli *et al.*, 1994), domestic stock (Moore *et al.*, 1991; Pépin *et al.*, 1995) and insects (Hughes and Queller, 1993). Moreover, the high levels of variability have been useful in the construction of genome maps (Love *et al.*, 1991; Morgante *et al.*, 1994) kinship and paternity analysis (Amos *et al.*, 1993; Pépin *et al.*, 1995; Tautz, 1989), phylogeny reconstruction (Bowcock *et al.*, 1994), clinical diagnostics (Thibodeau *et al.*, 1993; Trapman *et al.*, 1994) and detection of variability in populations which contain few or no allozyme polymorphisms (Hughes and Queller, 1993).

Finally, PCR amplification of short simple sequence loci, applied to degraded DNA obtained from museum collections (Ellegren, 1991; Hagelberg *et al.*, 1991) can be used to compare variation and relationships among extinct and contemporary populations. The utility of the PCR can be further applied to samples acquired by non-intrusive methods, such as the collection of excrement, sloughed skin (Amos *et al.*, 1992) and feather shafts (Ellegren, 1991).

2.1.4 Random amplified polymorphic DNA (RAPD)

The development of arbitrarily-primed PCR (Welsh and McClelland, 1990) or random amplified polymorphic DNA (Williams *et al.*, 1990) excited much interest among plant geneticists who found the application useful in the construction of linkage maps (Klein-Lankhorst *et al.*, 1991; Tulsieram *et al.*, 1992) and identification of crop plants (Hu and Quiros, 1991; Klein-Lankhorst *et al.*, 1991; Fukuoka *et al.*, 1992; Kaemmer *et al.*, 1992). Unlike other PCR-based strategies, RAPD analysis can be conducted in the absence of sequence information on the target DNA and in fact, may provide a useful means of investigating species about which there is little or no molecular information (Jones *et al.*, 1994).

Single decamer oligonucleotide primers of arbitrary sequence are used to amplify random fragments of DNA, generating a complex fingerprint (Williams *et al.*, 1990). Variations in these profiles arise due to length polymorphisms arising from point mutations in the primer binding sites which either facilitate or prevent primer binding. The technique provides the advantage of quickly and inexpensively generating many polymorphisms which may be read directly from agarose gels.

However, the limitations of RAPD analysis are such that accurate resolution of polymorphisms requires the use of a comprehensive set of controls (Smith *et al.*, 1994), determination of band homology (Stammers *et al.*, 1995) and careful interpretation of results. Furthermore, because almost all RAPD markers are dominant (Williams *et al.*, 1990), it is not possible to distinguish homozygotes from heterozygotes on a gel. This can only be overcome with the laborious task of using pairs of tightly-linked dominant markers, several co-dominant markers (Williams *et al.*, 1990; Tulsieram *et al.*, 1992) or segregant analysis (Hunt and Page, 1992).

The use of RAPD in animal studies is increasing. However, concerns about the appropriateness of the data for use in phylogenetic comparisons (Smith *et al.*, 1994) has restricted the application to generating markers for population differentiation (Kambhampati *et al.*, 1992), identification of domestic breeds (Gwakisa *et al.*, 1994) and determination of variation in populations displaying low levels of variability with other molecular markers (Puterka *et al.*, 1993; Bardakci and Skibinski, 1994).

2.2 STUDY AREA AND SAMPLE COLLECTION

2.2.1 Study area

2.2.1.1 Bottlenose dolphins

The area sampled for this species included the inshore regions of the east coast of South Africa, extending from Zinkwazi (29°11'S/31°26'E) to Maitland (34°02'S/25°38'E), a locality south of Algoa Bay (33°53'S/25°41'E) (Fig. 2.1). For the purpose of hypothesis testing, the study area was divided into two principal regions:

KwaZulu-Natal (28°48'S/32°06'E to 31°05'S/30°11'E) represents the section of coastline which is heavily netted to protect bathers from attack by large predatory sharks and includes more than 400 shark nets set at 45 bathing beaches between Zinkwazi and Mzamba (31°05'S/30°11'E) (Cockcroft, 1990b). This region was further subdivided into six "preferred areas" designated by Ross *et al.* (1987, 1989) and Cockcroft *et al.* (1990b). These areas include 1) Zinkwazi - Ballito Bay, 2) Ballito Bay - Durban, 3) Ansteys Beach - Karridene, 4) Umgababa - Southport, 5) Umtentweni - Southbroom and 6) Trafalgar - Mzamba (Fig. 2.1).

The East Cape (31°10'S/30°09'E to 34°00'S/23°30'E) represents a region which is unstressed by incidental shark net capture. Samples for this study were taken from the Wild Coast (31°12'S/30°05'E), Algoa Bay and Maitland (Fig. 2.1).

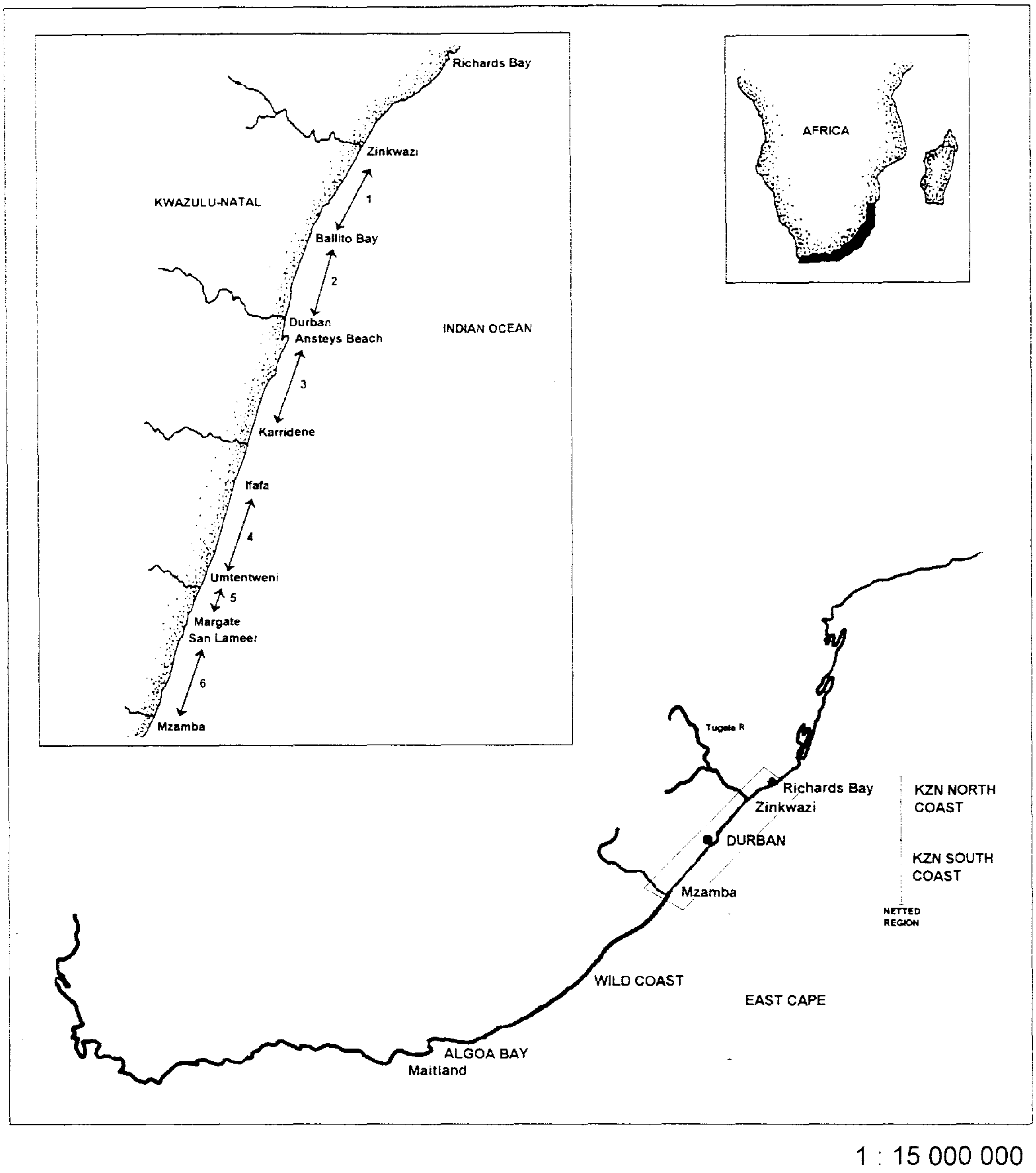


Figure 2.1. The South African east coast indicating localities from which bottlenose dolphins were sampled for this study. The small inset indicates the position of this region of the coast in relation to the rest of the southern African coastline. The sample area spans approximately 1000 km of coastline. The enlarged area indicates the region of the KZN coast protected by shark nets. Localities 1 to 6 represent the 'preferred areas' suggested by Cockcroft *et al.* (1990b) and span a distance of approximately 350 km.

2.2.1.2 Humpback dolphins

The principal study area for this species comprised the netted region from Richards Bay (28°48'S/32°06'E) on the northern KZN coast, to Margate (30°49'S/30°26'E) on the south coast (Fig. 2.2a), the extent of the East Cape coast sampled was largely determined by the opportunistic occurrence of stranded animals. In addition to the samples taken from shark nets, stranded animals were sampled from Maputo Bay (25°56'S/32°25'E), Algoa Bay and False Bay (34°15'S/18°40'E) (Fig.2.2a).

Humpback dolphin samples were also acquired from inshore waters around Hong Kong (Fig. 2.2b). Two samples were collected from localities in the New Territories (22°27'N/113° 57'E and 22°22'N/113°59'E), four from localities along the northern coast of Lantau Island (22°19'N/114°01'E to 22°15N/113°51'E), one from the southern coast of Lantau Island (22°13'N/113°55'E), two from Cheung Chau Island (22°13'N/114°02'E) and a single sample from the Soko Islands (22°09'N/113°55'E).

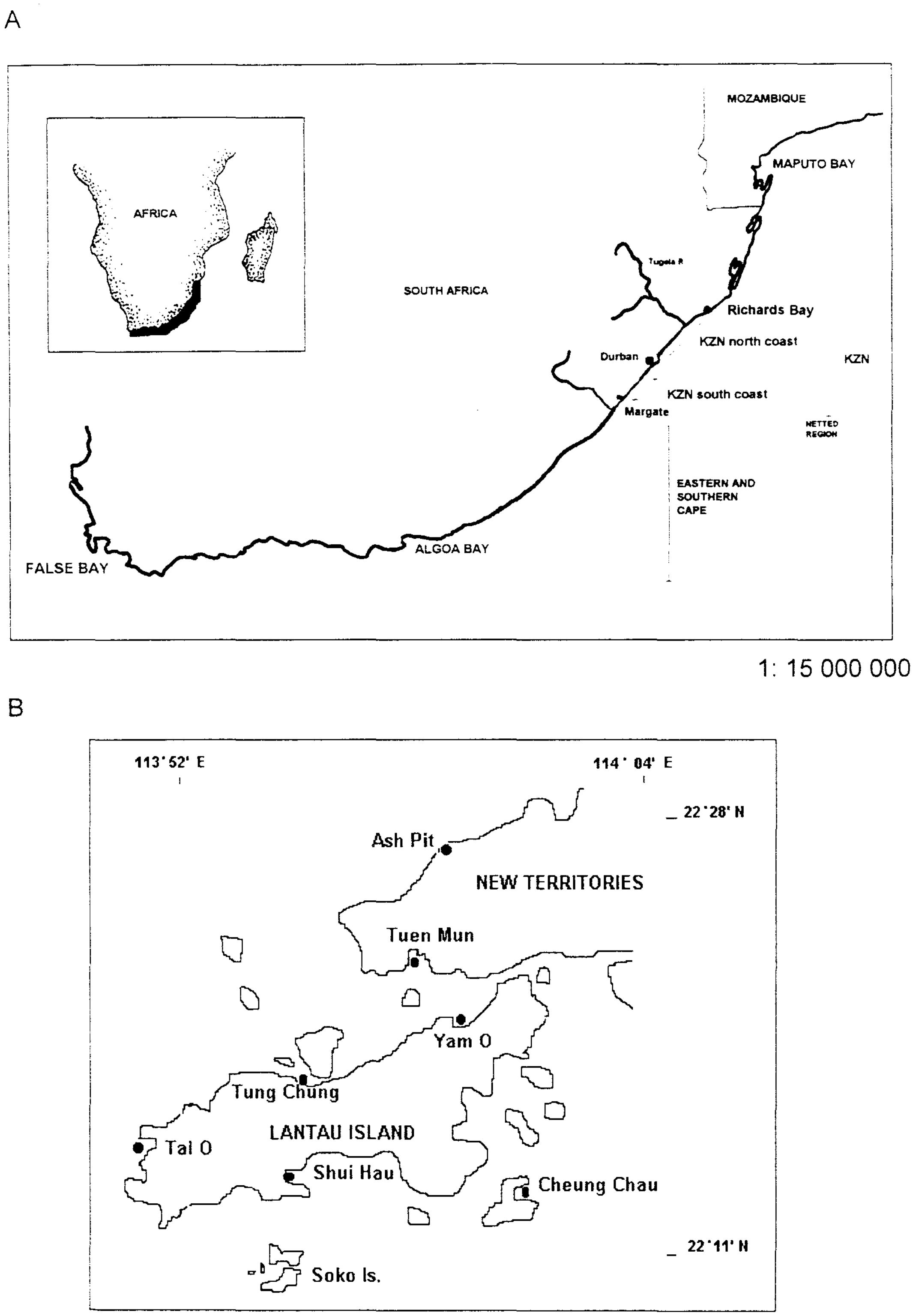


Figure 2.2a) The South African east coast indicating localities from which humpback dolphins were sampled for this study. The sample area, extending from Maputo Bay to False Bay, spans some 2000 km of coastline. The inset indicates the position of the sample area in relation to the rest of the southern African coastline **b)** Localities sampled around Lantau Island and the Hong Kong New Territories coastal region.

2.2.2 Sample collection

The collection of samples from shark nets poses the potential problem of obtaining a sufficient number of randomly sampled individuals, as both species demonstrate a demographic bias in the animals incidentally caught (Cockcroft, 1990b; Cockcroft and Ross, 1991). Furthermore, biopsy sampling of free-ranging schools of humpback dolphins proved almost impossible, due to the elusive nature of the species and the small average school size. Regular biopsy sampling for this study was restricted by the logistics of permit acquisitions, weather conditions and co-ordinated planning. Although the samples used in this study may not be sufficiently large or random to be ideally representative of the populations concerned, the KZN sample nonetheless comprises 5.9 % and 15 % of the estimated census population size of bottlenose and humpback dolphins, respectively.

2.2.2.1 Bottlenose dolphins

A total of 74 east coast inshore bottlenose dolphins were analysed in this study. Tissue samples were obtained from 41 bottlenose dolphins captured incidentally between 1988 and 1995 in shark nets set along the KwaZulu-Natal coast. Upon removal from the nets, dolphins were stored at -20°C pending necropsy, whereupon they were thawed and kidney, liver, muscle, heart and skin samples were taken. These were stored in liquid nitrogen during the earlier stages of the project, but were later moved to a -70°C freezer.

Individuals were sampled from free-ranging schools with a crossbow which fired a bolt modified to extract a small plug of skin. Sampling trips were conducted year-round between 1992 and 1995. Twelve bottlenose dolphins were sampled in this way in Algoa Bay, 11 in Durban and six samples were taken from a group moving off the former Transkei coast. Skin plugs were removed from the bolt and immediately stored in 95% ethanol. All biopsy samples were stored in 95% ethanol at -20°C upon return to the laboratory.

A skin sample was obtained from a bottlenose dolphin which had stranded near Maitland (Fig. 2.1) and blood was obtained from three unrelated captive inshore bottlenose dolphins from the Port Elizabeth Museum, each of whom originated from the Algoa Bay region. These samples comprised part of the Algoa Bay group (Fig. 2.1) in this study.

Blood was also obtained from a west coast offshore bottlenose dolphin kept at Sea World, Durban. The blood was stored in Vacutainers™ containing 2% K_2EDTA at 4°C for up to two weeks prior to DNA extraction. The D-loop sequence of an Atlantic Ocean offshore bottlenose dolphin was donated by Dr Barbara Curry (Southwest Fisheries Science Centre, La Jolla, CA).

2.2.2.2 Humpback dolphins

Incidental dolphin bycatch accounted for the majority of humpback dolphin samples used in this study. Tissue was collected from 30 humpback dolphins taken in shark nets on the KZN coast between 1988 and 1995. Collection and storage procedures were as described in section 2.2.2.1.

A single sample was obtained from a humpback dolphin in Algoa Bay. Kidney and heart tissue, retrieved from a stranded humpback dolphin in False Bay was donated by Professor Peter Best. These samples were stored at -20°C until analysis was conducted. Dried gum tissue adhering to the tooth of a humpback dolphin slaughtered in Maputo Bay was removed with a sterile scalpel and stored dry in a sterile Eppendorf tube. Skin samples taken from 10 stranded humpback dolphins in Hong Kong (samples courtesy of Lindsay Porter, University of Hong Kong) were stored in a saturated salt solution containing 20% dimethyl sulphoxide (DMSO) (Amos and Hoelzel, 1991).

2.2.3 Terminology

2.2.3.1 Bottlenose dolphins

In this study, a bottlenose dolphin *school* refers to a single group of animals in close association, engaged in travelling, feeding or interacting for the duration of the observation or sampling event. It is analogous to the groupings recognised by Wells *et al.* (1987), Scott *et al.*, (1990) and Würsig and Harris (1990) and identical to the *school* recognised by Weigle (1990). The term *group* is used in an *ad hoc* context and defines an artificially designated collection of samples for the purpose of analysis or discussion. It is also used to describe subsets of the population which are neither schools nor formal stocks. In view of a lack of knowledge of the population and stock boundaries of Southern African inshore dolphins, the term *population* is used in this study to describe all of the dolphins occurring off the coast of South Africa and makes no assumptions regarding population structure or differentiation. KwaZulu-Natal and the East Cape comprise the two *regions* into which the study area is divided. Bottlenose dolphins from either region are referred to as *stocks*; however, unless specified, the use of this term does not pre-empt the existence of structure or differentiation within or between the two regions. *"Preferred areas"* refers to hypothesised regions of residency, as suggested by Ross *et al.* (1987) and Cockcroft *et al.* (1990b) and is analogous to the term 'home ranges' used by Scott *et al.* (1990).

2.2.3.2 Humpback dolphins

The terms *school*, *group* and *population* are used in the context described in section 2.2.3.1. However, the Maputo Bay sample is not regarded as part of the South African population, but is considered within the southern African population. *Region* is used, in the context of the humpback dolphin, to describe, independently, 1) Maputo Bay, 2) the KZN north coast, encompassing all samples taken north of Durban, 3) the KZN south coast, including all samples and sites south of Durban and 4) the eastern and southern Cape, describing all localities from Algoa Bay, southwards.

2.3 DNA EXTRACTION

Where possible, kidney was preferred for DNA extraction. Skin was used before either muscle or heart tissue and liver was not used as a DNA source. DNA was extracted from skin and tissue samples acquired before 1993 by a method modified from Gold and Richardson (1991). Approximately 1g of tissue was crushed to a fine powder in liquid nitrogen with a mortar and pestle. The powder was suspended in 250µl STE buffer (0.1M NaCl, 50mM Tris, 1mM EDTA; pH 7.5), to which was added 20% SDS solution to a final concentration of 5%. The samples were then extracted once with an equal volume of buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1) and twice with an equal volume of chloroform:isoamyl alcohol (24:1). High molecular weight total genomic and extracellular DNA was preferentially precipitated following the addition of two volumes of ice cold isopropanol (Sambrook *et al.*, 1989). The DNA was washed once with 70% ethanol, air dried and resuspended in TE buffer (10mM Tris, 1.0mM EDTA; pH 8.0).

Samples acquired since 1993 were processed using a modification of the method of Hunt and Page (1992). This method obviated the sporadic occurrence of contaminating phenol in the purified nucleic acid. Approximately 0.5g of tissue was chopped finely with a scalpel blade on a sterile plastic Petri dish and placed in an Eppendorf tube containing 400µl CTAB extraction buffer (1% cetyltrimethyl ammonium bromide, 0.75M NaCl, 50mM Tris, pH 8.0, 10mM EDTA and 100µg/ml Proteinase K). Samples were incubated at 56°C for 2 hours and extracted twice with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with the addition of 1/10 volume of ammonium acetate and two volumes of ice cold isopropanol. The DNA was washed once with 70% ethanol, air dried and resuspended in TE buffer.

DNA was purified from blood using a method modified from Miller *et al.* (1988). The white blood cell buffy coat was pelleted from 200-500μl whole blood by a 10 second centrifugation at 1000 rpm. The cells were resuspended in 200μl TE buffer (10mM Tris, 1mM EDTA, pH 8.0) containing 0.5% SDS and 200μg/ml Proteinase K and incubated at 56°C for 2 hours. The digested protein was removed with the addition of 50μl of 6M NaCl, followed by centrifugation at 5000 rpm for 10 minutes. The DNA was precipitated by the addition of two volumes of ice cold isopropanol. The pellet was washed once with 70% ethanol, air dried and resuspended in TE buffer.

The dessicated sample and those stored in salt/DMSO were rehydrated overnight at 4°C in TE buffer prior to extraction using the modified method of Hunt and Page (1992). This method was further adapted by extending the CTAB/proteinase K incubation to 4 hours.

Approximate DNA concentration was quantified spectrophotometrically by measuring the absorbance at 260nm and by fluorescence after staining with ethidium bromide (Sambrook *et al.*, 1989). Working stocks of all samples were aliquoted from the purified DNA and kept at 4°C. The remainder of each sample was stored at -20°C.

2.4 PCR AMPLIFICATION OF mtDNA CONTROL REGION FRAGMENT

A 500 bp portion of the mitochondrial DNA control region, extending from the tRNA^{Thr} gene to the similarity block B, characterised for the Commerson's dolphin (Southern *et al.*, 1988) was amplified by the polymerase chain reaction using the primers L14841 (Kocher *et al.*, 1989) and H00034 (Rosel *et al.*, 1994) (Table 2.1; Fig. 2.3).

Amplification reactions of 100 µl contained 10-100 ng DNA, 10mM Tris-HCl, 10mM KCl, 1.5mM $MgCl_2$, 100µM of each dNTP, 0.3µM of each primer and 2.5 units of AmpliTaq® DNA Polymerase (Perkin-Elmer). Samples were amplified with a GeneAmp® PCR System 9600 thermocycler (Cetus Corporation), using 35 cycles of denaturation at 94°C for 45 seconds, annealing at 48°C for 60 seconds and extention at 72°C for 90 seconds. The amplification included an initial denaturation of 180 seconds and a final extention of 300 seconds.

2.5 DIRECT SEQUENCING OF mtDNA CONTROL REGION FRAGMENT

2.5.1 Taq Polymerase PCR sequencing

The control region fragment from 48 bottlenose dolphin samples and two humpback dolphin samples was cycle sequenced using Taq polymerase. Excess primers, salts and dNTPs were removed by filtration with Microcon-100 microconcentrators (Amicon). The dideoxy chain termination reaction (Sanger *et al.*, 1977) was performed on the heavy strand using primer L14841 (Kocher *et al.*, 1989) and on the light strand using H16498 (after Rosel *et al.*, 1994) (Table 2.1; Fig. 2.3) with the Applied Biosystems Inc. PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit.

Excess dNTPs. ddNTPs and primers were removed by centrifugation through Centri Sep spin columns (Princeston Separations) packed with Sephadex G-50 (Sigma). The eluted product was dried in a SpeedVac SVC 100 (Savant) and stored at 4°C until the sequenced products were separated using an Applied Biosystems Inc. 373A autosequencer. Electropherogram readouts were edited and aligned on a Mackintosh IIsi using the SeqEd software (ABI).

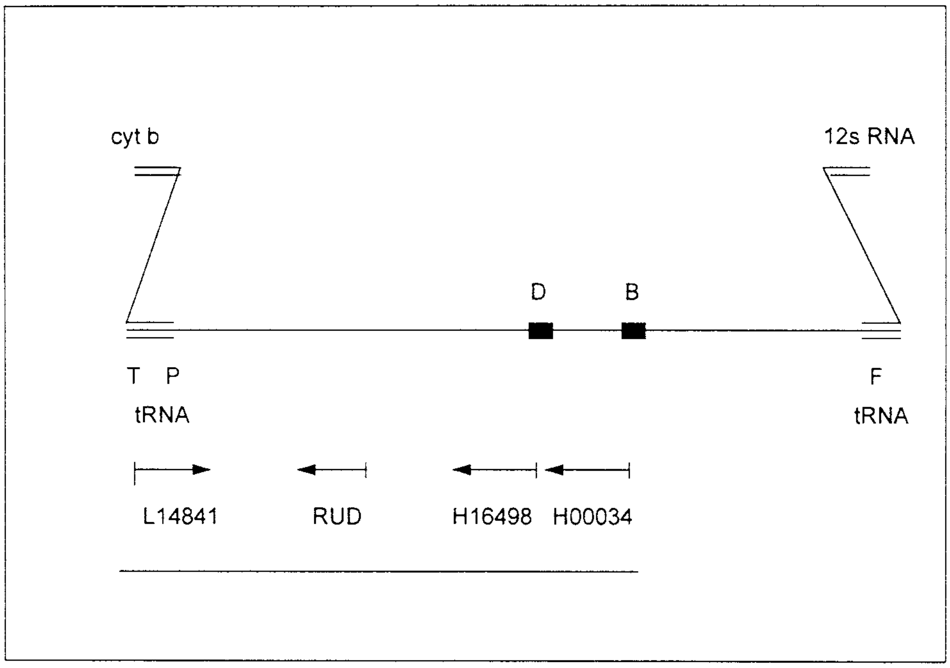


Figure 2.3 Primer positions within the section of the control region studied. D and B represent the similarity blocks B and D characterised by Southern *et al.* (1988).

2.5.2 Sequencing of PCR products with Sequenase^{TM} DNA polymerase

The remainder of the samples were sequenced using the USB Sequenase PCR Product Sequencing Kit (Amersham LIFE SCIENCE). Prior to sequencing, excess primers and extraneous single-stranded DNA were removed from the PCR reaction by incubation with exonuclease I and dNTPs were removed by incubation with shrimp alkaline phosphatase, as outlined by the manufacturer. The amplified control region fragment was labelled with [α-^{35}S]-dATP using a three-dNTP labelling method suggested in the user's guide and sequenced by means of the dideoxy chain termination reaction (Sanger, 1977). The light strand was sequenced using primer H16498 and a nested primer, RUD, designed from the sequences determined using the methods described in 2.5.1 (Table 2.1; Fig. 2.3). This improved the resolution of the sequence towards the 3' end of the fragment.

Products were separated on 6% and 8% denaturing polyacrylamide gels, made according to specifications outlined in the Sequenase PCR Product Sequencing Kit user's manual. X-ray film (Cronex, Protea Laboratories) was exposed to the fixed, dried gels for 36-144 hours and the sequences were read and aligned by eye.

Table 2.1. Primer sequences for the amplification and sequencing of the 500bp control region fragment.

	Primer sequence	Reference
PCR amplification	H00034 5'-TAC CAA ATG TAT GAA ACC TCA G-3'	Southern *et al.* (1988)
	L14841 5'-ACA CCA GTC TTG TAA ACC-3'	Kocher *et al.* (1989)
Sequencing	H16498 5'-CCT GAA GTA AGA ACC AGA TG-3'	Southern *et al.* (1988)
	RUD 5'-CGA GCT TTA ACT TAT CGT ATG G-3'	This study

2.6 MICROSATELLITE ANALYSIS

Primers defining six microsatellite loci (Table 2.2), isolated randomly from the long-finned pilot whale *Globicephala melas* (Schlötterer *et al.*, 1991; Amos *et al.*, 1993) were used to investigate length polymorphisms in nuclear loci. All of the primers flank simple sequence stretches of GT or GA dinucleotide repeats (Schlötterer *et al.*, 1991). The primer sequences are given in Table 2.2.

Amplification reactions of 10μl contained 10 - 100 mg DNA, 20 mM Tris-HCl (pH 8.4), 50mM KCl, 2mM $MgCl_2$, 100μM of each dNTP, 0.2mM of each primer and 2 units of Taq polymerase (GibcoBRL, Life Technologies). Each reaction was overlaid with 20μl of sterile mineral oil (Sigma). Amplification was conducted in an OmniGene thermocycler (Hybaid), using 35 cycles of denaturation at 95°C for 30 seconds, annealing at 40-44°C for 45 seconds (Table 2.2) and strand extention at 72°C for 45 seconds. An initial denaturation at 95°C for 120 seconds and a final extention at 72°C for 180 seconds was conducted.

Microsatellite alleles were resolved both autoradiographically and with a silver stain. In the former, one of each pair of primers was endlabelled with γ-^{32}P-dATP using the polynucleotide kinase reaction (Sambrook *et al.*, 1989). Following amplification, two microlitres of each product was mixed with 2μl of formamide loading dye and heated to 95°C before being loaded onto a 6% or 8% polyacrylamide gel containing 50% w/v urea. A sequencing reaction of M13 control DNA supplied with the sequencing kit used in 2.5.2 above, was used to generate a sequence ladder against which microsatellite allele sizes were measured.

Gels were fixed to the front gel plate with γ-methylacryloxypropyltrimethoxysilane (Sigma) to facilitate silver staining. Samples were prepared as described above and a sequencing reaction of the plasmid pGEM®-3Zf control DNA supplied with the Silver Sequence™ DNA sequencing system (Promega) was run adjacent to the samples to generate a sequence ladder against which allele sizes were measured. In some cases, the guanine termination reaction of the bottlenose dolphin mtDNA d-loop fragment was run adjacent to the microsatellites as a size marker. After electrophoresis, gels were silver-stained following the protocol of Bassam *et al.* (1991). Following fixation in 10 % acetic acid for 30 - 60 minutes, the gel was rinsed twice with ultrapure water. The gel was then incubated in a solution containing 1g/l silver nitrate and 1.5 ml formaldehyde for 30 minutes, after which it was rinsed twice with ultrapure water. The gel was developed by incubation in 30g/l sodium carbonate containing 2mg/l sodium thiosulphate. Development was terminated with the addition of 10% acetic acid. Developed gels were blotted onto Whatman No.1 paper and vacuum dried.

Table 2.2 Primer sequences and annealing temperatures for the six microsatellite loci studied (Schlötterer *et al.*, 1991; Amos *et al.*, 1993).

Sequence	Annealing temperature
Locus 199/200 5'-TGA AAT TCT TCA TCA GT-3' 5'-GTT AAT GTA GGC AGA CT-3'	44°C
Locus 415/416 5'-GTT CCT TTC CTT ACA-3' 5'-ATC AAT GTT TGT CAA-3'	40°C
Locus 417/418 5'-GTG ATA TCA TAC AGT A-3' 5'-ATC TGT TTG TCA CAT A-3'	40°C
Locus 464/465 5'-GGG GTT TCT CCT CTA-3' 5'-TGA TCT GCC AAT AAG A-3'	42°C
Locus 486/469 5'-ACC CCA GAG AAA ACA-3' 5'-CAA GGT ATT TCA GAA-3'	40°C
Locus 409/470 5'-GTT TTG GTT GCT TGA-3' 5'-TAA AAG ACA GTG GCA-3'	40°C

2.7 RAPD ANALYSIS

An array of 20 random primers was screened for reproducible fragment amplification and polymorphism. Ten were designed in the laboratory and the remaining 10 were commercial random oligonucleotides from Operon Technologies. Eight primers gave reproducible products with sufficient polymorphism for analysis (Table 2.3). A standard, optimised reaction mix was used in RAPD amplification. Reaction volumes of 20 μl contained 100 ng DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.5 μM primer and 2 units of Taq DNA polymerase (GibcoBRL, Life Technologies). Each reaction was overlaid with 20 μl of sterile mineral oil (Sigma). A standard temperature profile consisting of an initial denaturation at 95°C for 120 seconds, followed by 30 cycles of 95°C for 30 seconds, 37-40°C for 30 seconds, 72°C for 80 seconds and a final extension at 72°C for 180 seconds was used to amplify RAPD fragments (see Table 2.3 for annealing temperatures).

Amplification products were separated on 2 - 2.5% agarose gels at 120 V for 5 - 6 hours and visualised with ethidium bromide. Where greater resolution of bands was required, products were separated in 8% vertical, nondenaturing polyacrylamide gels (20 cm x 30 cm; 1mm thickness) (Laemmli, 1970) and silver stained as described in section 2.5.

2.7.1 Testing for reproducibility of amplified fragments

DNA samples extracted independently from skin and kidney from the same individual, were amplified simultaneously to test the reproducibility of the method with different DNA sources. Most samples gave identical fingerprints; however some of the slightly older kidney samples gave fingerprints with differing intensities or complete absence of fragments. Thus, only DNA originating from skin samples was used for subsequent analysis. Additional assessments of reproducibility included the amplification of duplicate, independently-extracted DNA samples and the inclusion of a DNA-free control sample at every round of amplification. Only consistent, strongly-amplified alleles were scored. Band intensity was not used as a scorable characteristic.

Table 2.3. Primer sequences and annealing temperatures used in the determination of RAPD polymorphism.

Primer sequence		Annealing temp. (°C)
Primer 1	5'-TCACATGGCA-3'	37
Primer 2	5' -TCGATGAACG-3'	37
Primer 3	5' -TGTCCCCGGC-3'	40
Primer 7	5'- ACGTAGCACT-3'	38
Primer 10	5'- AGTCACTGGT-3'	38
Primer 11	5' -GTGATGAAGG-3'	37
Primer 12	5'- ACCTGCGTTA-3'	37
OPA-11	5'- ACGGCGATGA-3'	38

2.8 GENDER DETERMINATION BY PCR

The PCR was used to determine the gender of unknown samples and free-ranging dolphins biopsied in this study. An attempt was made to use published primers targeting the SRY region of the male Y chromosome (Palsbøll *et al.*, 1992). However, the expected ~170 bp fragment consistently failed to amplify, despite changes in annealing temperature and differential primer concentrations. Careful examination of the primer sequences suggested that dimerisation of the primers may have caused the failure to amplify. This was characterised by 40% complementarity between the two primers (Table 2.4).

New primers designed from the published human and rabbit sequences (Sinclair *et al.*, 1990) (see Table 2.4) were successful in amplifying a ~220 bp fragment. Combinations of the two sets of primers revealed that the failure of the first set to amplify the SRY fragment was probably due to low sequence homology in the reverse primer, so that it showed greater affinity for the forward primer than for the target sequence. The most consistent positive results were obtained with primers 593 and 802 (Table 2.4), however, incomplete homology and the low copy number of the target region made definitive positive scoring difficult in some samples. The products from three amplification reactions for each of two bottlenose and one humpback dolphin males were sequenced using primers 607 and 802. As expected, the sequences were identical. Primers SRYJF and SRYJR (Table 2.4) were designed from the dolphin sequence and used in gender determination of both humpback and bottlenose dolphins. Males displayed the expected ~210 bp fragment as well as a larger (~300 bp) fragment. Females lacked the 210 bp fragment, while the larger fragment was consistently amplified. This served as an internal control, ensuring that absence of the 210 bp fragment reflected a true negative, rather than a failed amplification.

Table 2.4. Primers used in the determination of gender of bottlenose and humpback dolphins. Bases in bold indicate complementarity between the primers. Primers 593, 764, 607 and 802 were designed from the human and rabbit sequences (Sinclair *et al.*, 1990) and primers JF and JR were designed from the dolphin SRY region sequenced in this study.

Primer sequence	Annealing temp. (°C)	Product size(s)	Reference
SRY593 5' AAG CGA C**CC** **ATG** A**AC** GC**A** TT 3'	55		Pallsbøl *et al.*, 1992
SRY764 3' **GGT** **AC**G **TG**T C**T**C TCT TTA TG 5'	55	~170 bp	Pallsbøl *et al.*, 1992
SRY 607 5' GAA CGC ATT CAT GGT GTG GTC 3'	50		This study
SRY 802 3' GGA AGC AGC CTT CCA ATT TTA GG 5'	50	~220 bp	This study
SRYJF 5' GCT CTG CAG AAT CCC A 3'	52		This study
SRYJR 3' GCT CTG TTT ATG GGC CCG 5'	52	~300 bp/ ~210 bp	This study

PART I

CHAPTER 3

GENETIC DIVERSITY OF S.E. AFRICAN BOTTLENOSE DOLPHINS

3.1 INTRODUCTION

Consideration of the effects of bottlenose dolphin mortality from incidental bycatch in shark nets (Cockcroft, 1990b, 1992; Cockcroft and Ross, 1991) and natural predation (Cockcroft *et al.*, 1989a) as well as exposure to chemical pollutant contamination (Cockcroft *et al.*, 1989b, Cockcroft *et al.*, 1991a) (and see section 1.4.1) supports the opinion that bottlenose dolphins on the KwaZulu-Natal coast are subject to depletion pressure (Cockcroft *et al.*, 1990b). Furthermore, census estimates reveal that existing population numbers are low (Ross *et al.*, 1989; Cockcroft *et al.*, 1991b, 1992; Peddemors, 1995; refer section 1.4.2).

The threat of depletion of the bottlenose dolphin population in KwaZulu-Natal initiated interest in the genetic variability of the population and a preliminary analysis was conducted which investigated the level of allozyme variation in dolphins taken as incidental bycatch in the KZN shark nets (Goodwin *et al.*, 1996). Although average heterozygosity estimates were comparable to other cetacean values, the proportion of polymorphic loci was low (15%) and a maximum of two alleles were scored for variable loci (op cit.). In order to propose conservation and management policies based on genetic diversity, suggestions made by allozyme data required confirmation using molecular techniques which provide greater resolution of genetic variability.

3.1.1 Genetic diversity

Maintenance of optimal genetic diversity is a requisite for the survival and persistence of a population or species and the biological principles directing the gain and loss of diversity constitute some of the fundamental questions addressed by conservation biology. Evaluation of the existing genetic diversity of a species offers not only an indication of the life history, breeding systems and evidence of recent historical events, but it allows for inferences about the evolutionary potential and future survival of the species and its populations.

3.1.2 Effective population size

The effective size of a population (N_e) provides an important function in conservation by influencing the rate at which genetic variability is lost and inbreeding occurs (Lande, 1991, Waples, 1991), primarily by genetic drift (Milligan *et al.*, 1994). Small populations are most noticeably affected by the random mutation of alleles which may result in fixation of the homozygous condition (Amos and Hoelzel, 1992) and ultimately, a decline in genetic fitness. The effective size of a population is determined by a complex interaction of social and demographic parameters, such as the sex ratio, dispersal rate, breeding system and temporal fluctuations in actual population size.

The effective size of a population is the size of a hypothetical 'ideal' population which would behave genetically in the same way as the population under investigation and may be measured using either demographic or genetic data. Relationships between demographic parameters such as those mentioned above and the effective population size can be used to estimate the latter from the former (Crow and Kimura, 1970). Alternatively, changes in measures of genetic variation between generations can be used to estimate effective population size over short periods such as those spanning the duration of the study. The temporal method has been proposed for the estimation of effective population size from changes in allele frequencies in small populations where a loss of genetic variation has occurred (Waples, 1989, 1991).

Long term effective population size may be useful in providing insight into the historical variation and effects of population decline in a population or species and may be an important indicator of the extinction probability (Goodman, 1987). Kimura and Crow (1964) showed that, under selective neutrality, the expected heterozygosity at equilibrium is a function of effective population size and the mutation rate. Several similar models have been proposed; however, several factors affect the suitability of heterozygosity as an indicator of effective population size. The estimation requires knowledge of the mutation rate about which there is much dispute. In addition, it was found for a large number of species (Nei and Graur, 1984) that empirical data do not support the relationship proposed by Kimura and Crow (1964). Furthermore, large interlocus variance in heterozygosity exists and most studies do not compare sufficient loci to yield accurate and comparable heterozygosity values (Archie, 1985), making the estimated N_e unreliable. An alternative approach to estimating long-term N_e uses nucleotide diversity where the substitution rate and generation length are known (Wilson *et al.*, 1985). This method is appropriate in the absence of recombination and is, therefore, effective for use with mtDNA data (Avise *et al.*, 1988).

3.1.3 Objectives

The purpose of this chapter of the study is to determine the genetic diversity of KZN and East Cape bottlenose dolphins and to investigate the implications of that diversity, coupled with demographic data, on the fitness and persistence of the species off the KZN coast. This was undertaken by analysing the following genetic parameters:

i) Nucleotide diversity in a portion of the mtDNA control region,

ii) Variation at six microsatellite loci,

iii) Variation in random amplified polymorphic DNA (RAPD),

iv) Estimation of effective population size.

3.2 MATERIALS AND METHODS

3.2.1 Sample collection

Samples taken from 38 incidentally-caught bottlenose dolphins and 11 biopsied free-ranging bottlenose dolphins were used to estimate the genetic diversity of the species along the KwaZulu-Natal coast. Six biopsy samples collected from a school off the Wild Coast (Fig. 2.1), 12 biopsy samples collected in Algoa Bay, a single sample retrieved from a stranded animal at Maitland (Fig. 2.1) and three unrelated aquarium animals represented the East Cape region. A detailed description of the samples can be found in sections 2.2.1.1 and 2.2.2.1. Sequence variation was compared between the study samples and two offshore bottlenose dolphin samples which were used in phylogenetic reconstruction (see Chapter 4). One sample originated off the South African west coast and the second sequence was donated by Dr Barbara Curry (see section 2.2.2.1 for further detail).

3.2.2 DNA analysis

DNA extraction, amplification, sequencing and electrophoresis were conducted as described in sections 2.3 to 2.5. Microsatellite analysis was performed as described in section 2.6 and RAPD was amplified and electrophoresed as described in section 2.7.

3.2.3 Terminology

KwaZulu-Natal and the East Cape are the two *regions* under investigation in this chapter. The KZN coast is subdivided into the north and south coasts, with the division occurring at Durban. This subdivision has been maintained from previous studies which have divided the KZN coast on the basis of demographic differences (Cockcroft *et al.*, 1989b; Ross *et al.*, 1989; Cockcroft and Ross, 1990; Cockcroft, 1992). The coast is further divided into six 'preferred areas' (inset Fig. 2.1) designated from sighting data and aerial surveys (Ross *et al.*, 1987; Cockcroft *et al.*, 1990b). The East Cape has not been divided into subregions. The three *study groups* referred to in this chapter are the KZN north coast, south coast and the East Cape.

3.2.4 Data analysis

3.2.4.1 Nucleotide diversity

Nucleotide diversity was determined as the average number of nucleotide substitutions per site in a population (Nei, 1987) and was measured according to the relationship $\pi = \Sigma_{ij}(x_i x_j \pi_{ij})$ where x_i and x_j are the population frequencies of the *i*th and *j*th haplotypes and π_{ij} is the proportion of different nucleotides between the *i*th and *j*th haplotypes.

3.2.4.2 Microsatellite variation

Genetic diversity for either region and for the entire population was measured as the percentage of polymorphic loci (P), the mean number of alleles per locus (A), direct-count heterozygosity (H; Nei, 1978) and the inbreeding coefficient (F_{IS}, Wright, 1951,1965) using BIOSYS-1 [(Swofford and Selander, 1981), modified to facilitate jack-knifing of F-statistic values (Weir and Cockerham, 1984)]. Loci were considered polymorphic if the frequency of the most common allele did not exceed 0.95. The mean number of alleles per polymorphic locus was also determined (sensu Hartl and Pucek, 1994). Allele frequency values were used to calculate observed and expected heterozygosity estimates. Conformance to

Hardy-Weinberg expectations was tested using Levene's (1949) correction for small sample size. Where deviations occurred as a result of the presence of rare alleles, the χ^2 test with pooling (Hartl and Clarke, 1989) was used. Multi-locus deviation from random mating was estimated using $F_{IS} = 1 - H_{obs}/H_{exp}$, where H_{obs} and H_{exp} are the observed and expected heterozygosity estimates, respectively. The significance of the inbreeding coefficient was tested using $\chi^2 = F^2N$, df = 1, where N is the mean sample size across all groups (Li and Horvitz, 1953). Chi-square values and *df* were summed over all loci and populations to determine the significance of total F_{IS}.

3.2.4.3 Long term effective population size

Long term effective population size (N_e) was estimated using the relationship $N_e = dx\ 10^6 / sg$, where *dx* is the average nucleotide diversity over all pairwise comparisons, *s* is the nucleotide substitution rate per million years and *g* is the average generation length in years (Wilson *et al.*, 1985, Waples, 1991).

3.2.4.4 Short term effective population size

Allele frequencies were used to calculate the short term effective population size (N_e) using the temporal method of Waples (1989, 1991). N_e can be estimated by $t / [2(F - 1/S)]$, where *t* is elapsed time in generations, *S* is the harmonic mean of S_0 and S_1, the number of individuals sampled in generations 0 and 1 and F is the standardised variance of allele frequency change. F is determined for each locus by $F = [(1/L-1) \Sigma^{L}_{i=1} (X_{0i} - X_{ti})^2] / [(X_{0i} + X_{ti}) / 2]$, where *L* is the number of alleles at a locus and X_{0i} and X_{ti} are the frequencies of allele *i* at generations 0 and *t*. F values from several individual loci were combined to produce a single mean F, using $F = \Sigma [(L_j - 1) F_j] / \Sigma (L_j - 1)$, where L_j represents the total number of alleles and F_j is the total F over *j* alleles (Waples, 1991).

3.2.4.5 RAPD data analysis

Consistent, reproducible bands were scored as present or absent and were assigned the value of 1 or 0, respectively. Allele frequencies of polymorphic bands were determined using RAPDBIOS (Black, 1996) and BIOSYS-1 (Swofford and Selander, 1981).

3.3 RESULTS

3.3.1 Mitochondrial D-loop sequence variation

The DNA sequence was resolved for the first 400 bp of the noncoding mitochondrial DNA control region, extending from the tRNA^{PRO}, but excluding it, to similarity block E in the conserved central region (Brown *et al.*, 1986; Southern *et al.*, 1988). All of the variation was detected in the variable region upstream of the central region. One insertion-deletion mutation and 28 substitutions, 25 of which were transitions and three transversions, characterised 13 haplotypes in 76 bottlenose dolphins studied. Excluding outgroups, fifteen variable sites defined 11 haplotypes in South African east coast bottlenose dolphins. If one sample (designated haplotype 5) is excluded, the number of variable sites detected among the remaining 73 samples is reduced to eight (Table 3.1). All of the mutations detected among samples of the ingroup were substitutions and all were transitions (A‹–›G = 9, C‹–›T = 6; excluding haplotype 5: A‹–›G = 6, C‹–›T = 2). Haplotypes 2 and 4, which accounted for 89 % of the inshore sample (Table 3.1), differed by a single A to G transition at position 392, while eight unique haplotypes differed from the consensus sequence by no more than two transitions. A single deletion was detected at position 131, relative to the two outgroup samples. Nucleotide diversity (π, Nei, 1987) was estimated at 0.001 substitutions per nucleotide site for KZN samples and 0.00075 substitutions for East Cape dolphins, with an average nucleotide diversity of 0.12% for the whole study area.

Table 3.1. Eight variable sites defined 10 haplotypes in South African coastal bottlenose dolphins. Haplotype 5 is suspected to represent an east coast offshore bottlenose dolphin (see text). Haplotypes 12 and 13 represent, respectively, the offshore form of *T. truncatus* from the west coast of South Africa and US Atlantic Ocean and are used as outgroups for phylogenetic analysis. The numbers of individuals from each locality displaying certain haplotypes are given. Dots indicate sites which are identical to those in the consensus sequence and the dash indicates a nucleotide deletion. Nucleotide position is recorded from 5' to 3' on the mtDNA heavy strand (similarity block E to tRNA^{PRO} in Southern *et al.* (1988)).

Haplotype #	Nucleotide position	Locality			
	111 1111111111 112222333 1113447001 1122222233 592559089 1233679020 3501346918 581342522	KwaZulu-Natal		East Cape	
		North coast	South coast	Transkei	Algoa Bay
4	GATGGATCAA CAGAGAAG-T TAATATGTA	17	13	3	4
2	....G	5	10	3	11
1	.........G				1
3	.........G ....G				1
9	....G......		1		
10	.......T..		1		
6	.......T.. .G........	1			
8	.......T.. ....A..	1			
7	....AG......		1		
11	....G.....C		1		
5	...AA.C..G T.A....... CG.C.....	S.A. east coast offshore			
12	T...A...G. T..GAG..T. C..CGC...	S.A. west coast oceanic			
13	.GC..T.T.. T...A.G.TA C.G.GCAC.	U.S. Atlantic oceanic			

3.3.2 Microsatellite variation and allele frequency differences

Bottlenose dolphins displayed only moderate polymorphism in microsatellite markers. Of the six microsatellite loci tested, only 66.7% and 50% were polymorphic in the KZN and East Cape samples, respectively. Observed heterozygosity ranged from 0.262 - 0.326 (Table 3.3) with a mean of 0.290 for the whole coast.

Allelic diversity was relatively low with a maximum of four alleles resolved for polymorphic loci (see Tables 3.2 and 3.3). Three of the four polymorphic loci revealed only two alleles, while locus 199/200 was characterised by four alleles displaying a unimodal distribution which was skewed to the left (Fig. 3.1).

In all four polymorphic loci, the tendency was toward amplification of longer alleles which occurred at greater frequency than shorter alleles (Fig. 3.1). Allele frequencies showed little difference between the designated study groups (Fig. 3.1). No private alleles were scored; however, alleles 166, 176 and 204 (locus 199/200) were present in the overall population at frequencies of 0.1 or less. Fixation of a single allele (192) was observed for locus 199/200 in the East Cape group (Fig. 3.1c).

3.3.3 Test for inbreeding and conformance to Hardy-Weinberg Equilibrium

Deviations from Hardy-Weinberg equilibrium were noted for all loci (Table 3.4). The KZN north coast displayed a significant excess of individuals homozygous for allele 256 at locus 415/416 and an over-abundance of heterozygotes at locus 464/465, while an excess of heterozygotes was indicated in both 464/465 and 409/470 in the East Cape. The presence of rare alleles at locus 199/200 caused a large deviation from random mating expectations in the KZN south coast.

However, conformance to equilibrium was observed using a χ^2 test which pooled genotypes into 1) homozygotes for the most common alleles, 2) common/rare heterozygotes and 3) rare homozygotes and other heterozygotes (Hartl and Clarke, 1989). The East Cape sample displayed fixation for allele 192 at locus 199/200. In addition, a significantly large inbreeding coefficient (F_{IS} = -0.422) was recorded, implying a deviation from random mating in samples from that region. No significant inbreeding was indicated in the two KZN groups (F_{IS}, Table 3.3) and overall F_{IS} (-0.203, χ^2= 1.018, $p>0.05$) gave no indication of significant deviation from random mating within the South African population as a whole.

3.3.4 Comparison with allozyme variability

A preliminary genetic study on KZN bottlenose dolphins, reported an average heterozygosity value of 0.073 $\pm$ 0.045 for 23 putative allozyme loci (Goodwin *et al.*, 1996). The observed heterozygosity (26%) was significantly greater (t = -8.33, $p << 0.01$) at the microsatellite loci studied (Table 3.5). Both methods revealed the highest levels of observed heterozygosity in the two southern-most designated groups (Table 3.5). A comparison of observed microsatellite heterozygosities from these groups and the East Cape reveals similar values of between 0.315 and 0.326 (Tables 3.3 and 3.5). Despite the difference in average heterozygosity estimated by the two methods, the average inbreeding coefficient (F_{IS}), calculated as a function of heterozygosity, produced very similar values (Table 3.5) implying a consistency in the data obtained from allozyme and microsatellite analysis.

Table 3.2. Allele sizes and frequencies of four polymorphic microsatellite loci (Amos *et al.*, 1993) in South African inshore bottlenose dolphins.

Locus	Size of alleles (bp)	Frequency of alleles
415/416	244	0.420
	256	0.580
409/470	186	0.430
	190	0.570
464/465	138	0.340
	140	0.660
199/200	166	0.014
	176	0.100
	192	0.850
	204	0.037

Table 3.3. Genetic variability at six microsatellite loci in South African coastal bottlenose dolphins, expressed as mean number of alleles per locus (A), mean number of alleles per ploymorphic locus (A_p), percentage of polymorphic loci (P) and average observed (H_O) and expected (H_E) heterozygosity. Standard errors are given in parentheses. Inbreeding coefficient (F_{IS}) is given by 1- (H_o /H_E) (see section 3.2.4.2). Asterisk (*) indicates significance at $\alpha < 0.05$.

	North coast (N=23)	South coast (N=31)	East Cape (N=18)
Mean number of alleles per locus (A)	2 (0.4)	2.2 (0.6)	1.5 (0.2)
Percentage of loci polymorphic (P)	66.7	66.7	50
Mean number of alleles per polymorphic locus (A_p)	2.5 (1.0)	2.5 (1.0)	1.75 (0.5)
Mean direct count heterozygosity (H_O)	0.262 (0.130)	0.281 (0.106)	0.326 (0.152)
Mean expected heterozygosity (H_E)	0.250 (0.090)	0.247 (0.089)	0.226 (0.103)
Inbreeding coefficient (F_{IS})	-0.048	-0.138	-0.442
χ^2 test for significant inbreeding	$\chi^2 = 0.06$	$\chi^2 = 0.527$	$\chi^2 = 4.85$ *

Table 3.4. Chi-squared (χ^2) deviations from Hardy-Weinberg expectations in polymorphic microsatellite markers resolved for South African inshore bottlenose dolphins. Values for a chi-square test with pooling are given in parentheses (see section 3.2.4.2), df = degrees of freedom, p = statistical significance, asterisks denote significance at $\alpha < 0.05$. Hardy-Weinberg estimations were not determined for locus 199/200 for the East Cape sample as it displayed fixation for a single allele.

	North coast				South coast				East Cape			
	415	464	199	409	415	464	199	409	415	464	199	409
χ^2	5.81	9.89	11.4	0.10	0.304	3.68	63.26 (0.47)	0.69	1.34	5.24	-	0.03
df	1	1	6	1	1	1	10 (1)	1	1	1	-	1
p	0.016*	0.002*	0.075	0.920	0.581	0.055	0.000* (0.493)	0.404	0.240	0.022*	-	0.032*

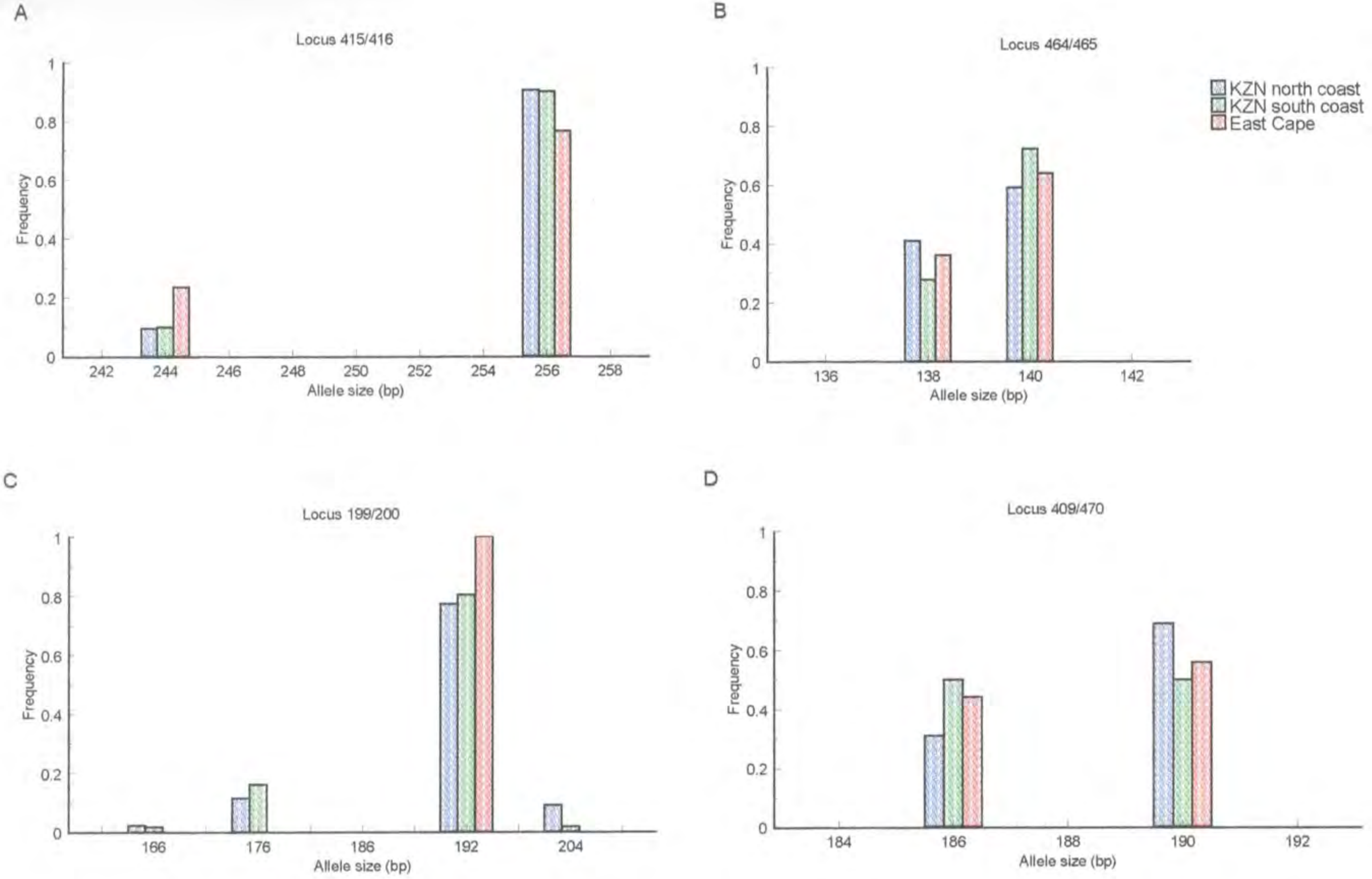


Figure 3.1 Allele frequency histograms for four polymorphic microsatellite loci in South African bottlenose dolphins.

Table 3.5 Genetic variability in KwaZulu-Natal bottlenose dolphins, determined for allozyme (Goodwin *et al.*, 1996) and microsatellite (this study) markers. The six groups correspond to those analysed in the former study, based on observational evidence for the subdivision of the KZN population into "preferred areas" (Cockcroft *et al.*, 1990b). A = number of alleles per locus; H_O and H_E = observed and expected heterozygosity estimates, respectively; F_{IS} = inbreeding coefficient. Standard errors are given in parentheses.

	Allozyme loci				Microsatellite loci			
Group	A	H_O	H_E	F_{IS}	A	H_O	H_E	F_{IS}
Zinkwazi-Ballito	1.2	0.044 (0.031)	0.050 (0.035)	0.012	1.8	0.272 (0.129)	0.329 (0.109)	0.173
Ballito-Durban	1.2	0.068 (0.041)	0.056 (0.032)	-0.210	1.5	0.211 (0.112)	0.226 (0.104)	0.066
Ansteys-Karridene	1.2	0.053 (0.038)	0.081 (0.044)	0.350	1.8	0.255 (0.147)	0.225 (0.102)	-0.133
Ifafa-Umtentweni	1.2	0.066 (0.037)	0.063 (0.036)	-0.050	2.0	0.204 (0.089)	0.220 (0.090)	0.073
Umtentweni-Margate	1.2	0.100 (0.056)	0.079 (0.043)	-0.270	1.7	0.315 (0.015)	0.271 (0.091)	-0.162
San Lameer-Mzamba	1.2	0.105 (0.066)	0.088 (0.048)	-0.190	1.8	0.323 (0.154)	0.245 (0.100)	-0.318
Mean	1.2	0.073 (0.045)	0.070 (0.040)	-0.042	1.76	0.263 (0.108)	0.253 (0.099)	-0.040

3.3.5 RAPD variation

Of the 20 random primers screened, three failed to amplify the DNA in 80% of the samples tested, five yielded inconsistent amplification products and 10 primers yielded monomorphic fingerprints. Only two of the 20 random primers screened produced sufficient consistently amplifiable, polymorphic fragments for analysis. Primers 7 and OPA-11 produced a total of nine scorable alleles in agarose gels. Of the six alleles scored for primer 7, four were present in 100% of the sample while the remaining two were found in 47.6% of the animals tested. The latter allele pair was encountered more frequently in samples from the KZN north coast and the East Cape than in samples from the KZN south coast (Fig. 3.2). One of the three alleles resolved for primer OPA-11 was present in 100% of the individuals. The remaining alleles occurred in 76.5% and 57.8% of the sample, respectively (Fig. 3.2).

In an attempt to resolve additional polymorphisms, amplified samples were separated on vertical nondenaturing polyacrylamide gels (see section 2.7). However, this resulted in the separation of as many as 25-30 bands, many of which were artifactual and confounded the true presence and absence of fragments (see section 6.3.7 for further explanation). No further analysis was conducted using RAPD data.

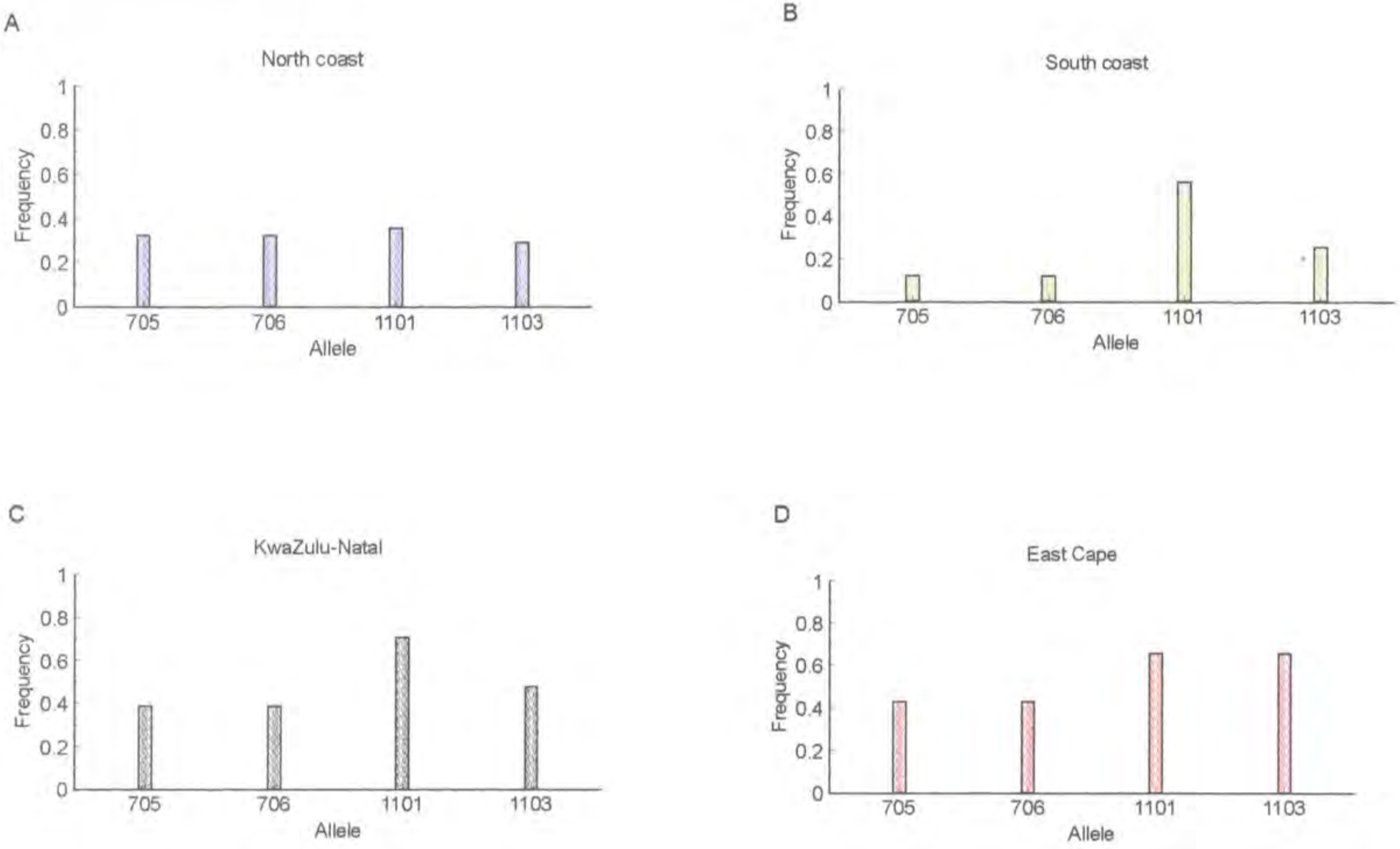


Figure 3.2 RAPD allele frequencies for polymorphic alleles of primers 7 and OPA-11. Histograms represent frequencies at which an allele was present. Alleles are designated by primer name, followed by allele number. Combined frequencies for the KZN north and south coasts are represented by (c).

3.4 DISCUSSION

3.4.1 Mitochondrial DNA diversity

A comparison of similar genetic studies reveals that the mtDNA nucleotide diversity within South African bottlenose dolphins in this study is lower than analogous values reported for other cetaceans and large mammals. Mitochondrial DNA diversity measures in other odontocetes, including the delphinid striped (*Stenella coeruleoalba*) and common (*Delphinus delphis*) dolphins, cover a wide range of values (0.18 to 1.4 %), all of which are substantially higher than the 0.12 % estimated for bottlenose dolphins in this study (Table 3.6). Furthermore, nucleotide diversity estimated for humpback (*Megaptera novaeangliae,* Baker *et al.*, 1993) and minke (*Balaenoptera acutorostrata,* Palsbøll *et al.*, 1995) whales from both hemispheres were six to twenty times greater than that determined for southern African bottlenose dolphins.

Of all the reported cetacean nucleotide diversity estimates, those presented for the narwhal approximate South African bottlenose dolphin values most closely. Palsbøll *et al.* (1994) reported a nucleotide diversity of 0.15 % estimated for narwhals from East and West Greenland and the eastern Canadian archipelago. Haplotype frequencies displayed a similar distribution, with two haplotypes accounting for the majority of the sample and the remaining haplotypes represented by single specimens (op cit). Only the vaquita reveals a lower level of nucleotide diversity than the bottlenose dolphin. No differences were found in 25 animals sequenced for the same gene region (Rosel and Rojas-Bracho, 1993).

The level of variation displayed in the maternal lineage of bottlenose dolphins in this study may have resulted from one or more of several factors. Low mtDNA diversity may be a result of recent excessive exploitation and a sudden decline in population number. Since the first installations off Durban, in 1952, over 400 gill nets have been set at 45 bathing beaches off the KZN coast (Cockcroft, 1990b; Cockcroft and Ross, 1991; Peddemors *et al.*, 1991; Peddemors, 1995). For the first 30 years, the nets were maintained by private tenders, making an assessment of catches difficult (Cockcroft and Ross, 1991). However, given the recorded catch statistics to date, it has been speculated that between 1000 and 2000 bottlenose dolphins have been caught since the nets were first installed (Cockcroft, personal communication). This at least equals the number of calves born during that period, assuming an annual growth rate of 3-5% (Reilly and Barlow, 1984), suggesting that over four and a half decades of net captures have likely resulted in depletion of the population.

However, low mtDNA diversity may merely be reflecting the evolutionary history of the species off this coast. As has been suggested as the cause of low nucleotide diversity in North Atlantic narwhals (Palsbøll *et al.*, 1994), evolution from a small founding population may be the primary cause of the observed level of diversity in bottlenose dolphins. Given that the south east African coast represents the edge of the Indo-Pacific range of the species, it is possible that the existing population arose from a small founding population originating further north.

However, a decline in nucleotide diversity may have occurred as a result of a population bottleneck. However controversial, diversity in the maternal genome of the two subspecies of cheetah (*Acinonyx jubatus jubatus* and *A. jubatus raineyi*) has been estimated at 0.182 % (Menotti-Raymond and O'Brien, 1993). The authors suggest that the cheetah experienced a severe bottleneck during the late Pleistocene (approximately 10 000 years ago) when a major extinction of large vertebrates occurred. They propose that the decline markedly reduced genetic variability (O'Brien *et al.*, 1983, 1985) and that the current level of variation represents subsequent mutation in the more rapidly evolving genomic elements.

A hypothesis may be considered for the nucleotide diversity found in the bottlenose dolphin. During the quarternary ice ages (10 000+ years BP) the sea level declined on the east African coast, severely reducing the area of the shallow inshore zone (see section 4.4.2.2). This may have caused repeated, rapid declines in the numbers of coastal dolphins and consequently, a loss in genetic variability.

An assessment of the three possibilities suggests the latter to be the most likely cause of low variability. High capture pressure off the KZN coast, which represents only a portion of the study area, is unlikely to have caused such low variability over the length of the south east coast over a period of 40 years. Second, no evidence is available to indicate that the south east African population was founded from populations further north. However, further study would be required to exclude this possibility. Given the geological history of the coast and possible timing of a bottleneck, it is most likely that a decline in the number of coastal dolphins with a change in sea level is responsible for a genetic bottleneck.

3.4.2 Microsatellite variation

Microsatellite variation was lower than expected for a potentially highly mobile marine mammal species. In fact, variability measures reveal levels of variation similar to those found in small, isolated populations or species (Table 3.7). The northern hairynosed wombat (*Lasiorhinus krefftii*) and the Ethiopian wolf (*Canis simensis*) have both undergone recent bottlenecks in genetic variability as a result of dramatically declining numbers and both are currently regarded as endangered species (Taylor *et al.*, 1994; Gottelli *et al.*, 1994). These species display levels of heterozygosity of between 20 and 30% and fewer than 2.5 alleles per locus, values which compare closely with those for the bottlenose dolphin in this study.

By contrast, a study of the long-finned pilot whale (*Globicephala melas*) revealed a minimum of three alleles per locus for the same six loci studied (Amos *et al.*, 1993). Locus 468/469, which was monomorphic in bottlenose dolphins, displayed 54 alleles in the pilot whale, permitting a comprehensive paternity assessment of the pod (op cit.). An average of eight alleles per locus and a mean heterozygosity of 74.5 % was scored for eight microsatellite loci in two discrete breeding colonies of grey seals (*Halichoerus grypus*) (Allen *et al.*, 1995), while average expected heterozygosity in humpback whales was estimated at 0.721, with an average of over 12 alleles scored for four microsatellite loci (Valsecchi *et al.,* 1997). These levels of variation are comparable to those of large terrestrial mammals and other marine species capable of extensive movement (Table 3.7).

These comparisons imply that the microsatellite variation observed in bottlenose dolphins is not only far lower than expected for the species, but may indicate an overall loss of genetic variability in the population. Alternatively, the large proportion of monomorphic loci and low variation may be an artifact of the genetic distance between the study species and that from which the loci were characterised. Moore *et al.* (1991) found that of the primers designed for cattle microsatellite loci only 56 % and 6.2 % would amplify sheep and horse DNA, respectively. Furthermore, only 42 % of the primers yielded polymorphic products in sheep and none of the products was polymorphic in horse DNA. Pépin *et al.* (1995) found that only 40% of the microsatellites isolated from cattle were useful in the study of goat DNA.

However, although genetic variation was found to be significantly lower for loci assayed from non-source species in marine turtles, mean heterozygosity in excess of 60 % and three to 15 alleles were recorded with intergeneric primers (FitzSimmons *et al.*, 1995), despite the source and non-source species representing over 300 million years of divergent evolution. Moreover, Roy *et al.*

(1994) reported allelic diversity of 4.5 to 6.4 alleles per locus and mean heterozygosities of 52 to 73 % for several species of wolf-like canids, using microsatellite loci identified from a domestic dog genomic library, while primer pairs designed from the domestic cat produced similar levels of variation in five other felid genera (Table 3.7). Primers designed from the harbour seal (*Phoca vitulina*) sequence produced eight distinct alleles and heterozygosity values in excess of 80 % in grey seals (*Halichoerus grypus*; Allen *et al.*, 1995).

Moreover, Schlötterer *et al.* (1991) indicated unusually low levels of divergence in the flanking sequences of the six microsatellite loci used in this study with sequence differences of 1% between the source species and *Tursiops truncatus* and an average difference of only 3.2 % between baleen and toothed whales. When four of the loci (415/416, 464/465, 199/200 and 417/418) were tested on randomly-selected samples of two baleen whale species, the fin whale (*Balaenoptera physalus*) and the minke whale (*B. acutorostrata*), seven of the eight tests revealed quantifiable polymorphism, implying that sequence conservation in the flanking sites was sufficient to assess microsatellite variability in other cetacean species (Schlötterer *et al.*, 1991).

From the examples given above, it would appear that the genetic distance between the long-finned pilot whale, *Globicephala melas* and the bottlenose dolphin, *Tursiops truncatus* should be sufficiently small to detect high levels of polymorphism in the latter. Characterisation of a large number of species-specific loci may assist in maximising the amount of detectable variability, however, the correspondingly low variation detected in functional allozymes (Goodwin *et al.*, 1996), noncoding mtDNA and randomly-primed DNA (this study), suggests that this would not result in a substantial increase in resolvable microsatellite variability. This suggests that the low variation found may well be a reflection of overall low genetic variability in the population.

3.4.2.1 Heterozygosity as a measure of variability

The use of heterozygosity estimates as a means of comparing genetic variation between and assessing genetic fitness within populations is well documented (Allendorf, 1986; Nei, 1987; references in Table 3.7). However, concerns have been raised about the validity of comparisons of heterozygosity, based on the analysis of an insufficient number of loci. Nei (1978) questioned the confidence with which true differences in heterozygosity are detected, given the limited number of loci assayed in most studies. Archie (1985) examined empirically, the number of independent loci needed to accurately compare species or populations on the basis of their level of heterozygosity and demonstrated that over large ranges of heterozygosities, the variance of the mean would be such that it would be necessary to examine a large number of loci to ensure the detection of all significant variability. Thus, the use of only four polymorphic loci in determining the nuclear diversity in bottlenose dolphins may have resulted in an under-estimation of microsatellite variability and should possibly be interpreted with caution. Most microsatellite analyses involving natural populations, however, do not yet permit the routine examination of 40 to 50 loci and most published studies, in which heterozygosity levels have been used to infer genetic variation in a population or species examined between six and 16 loci (see references in Table 3.7). Moreover, the genetic variation of a population or species is determined by several components, of which heterozygosity is only one. In this study, the low heterozygosity estimate was supported by few alleles at a microsatellite locus, a parameter which provides a more accurate indication of a reduction in genetic variation (Nei *et al.*, 1975).

3.4.2.2 Departure from Hardy-Weinberg Equilibrium

The departures from Hardy-Weinberg equilibrium (HWE) noted in this study are apparently not uncommon for microsatellite loci (Bruford and Wayne, 1993). Heterozygote deficiencies may be explained by difficulties in discriminating between homozygotes and heterozygotes (Chakraborty *et al.*, 1992) and the presence of null alleles resulting in pseudo-homozygotes (Bruford and Wayne, 1993), as well as the effects of population subdivision (Devlin *et al.*, 1990).

Fixation of allele 192 in the East Cape subpopulation may be explained in a number of ways: the absence of heterozygotes at this locus could merely be the result of a low number of heterozygotes in the South African population as a whole, as suggested by the low frequency of alternative alleles at this locus (Fig. 3.1; Table 3.2). The homozygote fixation may also represent the existence of a null allele at this locus. However, coupled with the heterozygote excesses in loci 409/470 and 464/465, homozygote fixation may indicate a deviation from random mating, as suggested by the large inbreeding coefficient (F_{IS} = -0.442) recorded for East Cape bottlenose dolphins.

The deviation noted may, alternatively, be the result of a sampling bias. Seventeen of the 18 individuals comprising the East Cape sample analysed for microsatellite variation were sampled by means of biopsy from one of only three independent schools. The first assumption was that the samples taken were from related family members which may have co-occurred in a school. However, gender determination (Section 2.8 and Chapter 5) indicated that 16 of the 17 animals sampled from free-ranging schools in the East Cape were male. In that case, two possibilities exist: either, related males co-occur in schools or the animals sampled were, in fact, unrelated and the schools sampled in Algoa Bay do display a significant level of inbreeding. It is difficult to speculate on which is the more likely explanation, however, previous field studies on coastal bottlenose dolphins elsewhere indicate that male groups comprise individuals who, as calves born to the same female group, form lasting associations with their contemporaries. These males are often genetically related, by virtue of the familial relationships of their mothers (Scott *et al.*, 1990). However, there is currently only circumstantial evidence to suggest the occurrence of all-male schools in KZN (Cockcroft and Ross, 1990) and no supporting evidence that members of all-male schools are related. If, however, there are familial relationships among the males sampled, then this study may under-estimate the amount of genetic variability in the East Cape sample. Without the analysis of additional samples from the East Cape, an accurate assessment of the factors influencing the departure from HWE is not possible.

3.4.2.3 Comparison with allozyme variation

Polistene wasps, characterised by low allozyme variation, have displayed levels of microsatellite variation comparable with large, outbred species (Hughes and Queller, 1993; Table 3.7). Although average heterozygosity estimates were higher in microsatellite loci than in the allozyme loci studied for bottlenose dolphins, the overall level of microsatellite variation was not comparable with that reported for other marine mammal populations. Furthermore, both molecular systems displayed low levels of polymorphism and few alleles per locus, both of which are indications that variation has been severely reduced (Nei *et al.*, 1975).

An interesting congruence was noted for allozyme and microsatellite variability measures. Both systems indicated greater levels of heterozygosity in the two southern-most groups. Furthermore, these values approximate those found in the East Cape sample. The two southern-most 'home ranges' represent the region on the south coast which is most heavily populated by bottlenose dolphins during the annual winter 'sardine run' (Chapter 5), many of which are suspected to be migrants from the adjacent East Cape coast (Goodwin *et al.*, 1996). It is possible that the elevated variation in the lower KZN south coast sample reflects not only the presence of migrant dolphins on the KZN coast, but also the genetic difference between bottlenose dolphins on the KZN and East Cape coasts (refer to sections 5.3.2 and 5.3.5).

3.4.3 RAPD variation

Low RAPD variation confirms the low variation seen in allozyme and microsatellite loci and the mtDNA control region. A similar level of diversity was reported for two populations of harbour seals, one small population in the Dutch Wadden Sea, which has suffered two recent incidents of population decline and a large outbred population on the coast of Scotland (Kappe *et al.*, 1995). Of the 50 primers tested, 20 generated amplified fragments, of which only two primers revealed polymorphism (op cit). Low levels of RAPD diversity in bottlenose dolphins in this study and in harbour seals in the Wadden Sea, may be explained by evolutionary genetic factors affecting small populations suspected to have undergone a population bottleneck. However, similarly low RAPD variation in a large, apparently diverse population (Kappe *et al.*, 1995) prompts speculation that the RAPD variability characteristic of plants, insects and fish does not occur in marine mammals.

Alternatively, considering the frequency with which primers failed to amplify the DNA or generate reproducible fragments, it is possible that the bottlenose dolphin DNA used for RAPD analysis was of insufficiently good quality. Most studies relying on the resolution of RAPD markers make use of template DNA isolated immediately after the onset of cell death (Chapco *et al.*, 1992; Hunt and Page, 1992; Bardakci and Skibinski, 1994; Fukatsu and Ishikawa, 1994), cloned bacterial DNA (Welsh and McClelland, 1990; Rasmussen *et al.*, 1994; Smith *et al.*, 1994) or DNA from almost unlimited plant material (Fukuoka *et al.*, 1992; Hu and Quiros, 1991; Hsiao and Rieseberg, 1994; N'Goran *et al.*, 1994). In all cases, good quality template DNA is ensured. Bottlenose dolphins used in this study were retrieved from shark nets up to 24 hours post mortem. In many cases, carcasses were frozen and thawed before samples were taken for DNA analysis. The release of nucleases shortly after cell death would doubtless cause some degree of degradation of high molecular weight DNA. Although specifically-targeted DNA would remain amplifiable, random degradation would almost certainly have an adverse effect on the amplification of arbitrarily-primed DNA. This suggests that DNA collected from stranded or incidentally-caught marine mammals is probably not suitable for reliable large scale studies using RAPD analysis. In addition, the difficulty with ensuring reproducibility of amplification products and the inability to determine the identity of a locus and its alleles, makes RAPD an unreliable and not very informative method of analysing genetic variation in natural cetacean populations.

3.4.4 Effective population size

3.4.4.1 Long term effective population size (whole sample)

The mtDNA nucleotide diversity of a population can be used to estimate the long term effective population size, given the mutation rate of the DNA and the generation time of the species (Wilson *et al.*, 1985; Waples, 1991). Female bottlenose dolphins reach sexual maturity at between nine and 11 years of age (Cockcroft and Ross, 1989). If the generation time is defined as the age of first reproduction, then an average generation for bottlenose dolphins would span approximately 10 years. An accurate estimation of the nucleotide substitution rate is required to ensure a reliable calculation of N_e. The rate of evolution of marine mammal control region mitochondrial DNA appears to vary considerably among species (Hoelzel *et al.*, 1991b; Hoelzel *et al.,* 1993; Wang *et al.*, 1996). Hoelzel *et al.* (1991b) and Baker *et al.* (1993) estimated the average substitution rate for the cetacean mitochondrial control region to be between 0.5 % and 1.0% per million years, while that of elephant seals appears to be greater at 6.0% per million years (Hoelzel *et al.* (1993). Using the range estimated for cetaceans, given a mean nucleotide diversity of 0.0012 and an average divergence of 0.121% for the whole population (section 4.3.1), the expected female effective population size ($N_{e(f)}$) for bottlenose dolphins is estimated to be between 11 800 and 23 600. This equation assumes a lack of population differentiation (Waples, 1991), thus any subdivision of the population or migration into the study area, both currently and historically, would bias the estimate. Considering that some structure does exist in the maternal lineage of bottlenose dolphins along the South African coast (see Chapter 5) and assuming that the study population is not closed to migration from the north and/or south, these figures will represent an inflated estimate of the long term $N_{e(f)}$. Nonetheless, it is still considerably smaller than $N_{e(f)}$ estimated for other cetacean species. Hoelzel and Dover (1991b) computed an $N_{e(f)}$ of 56 000 for populations of minke whale using a mutation rate of 0.5 % per MY, while using published estimates of nucleotide diversity, long term effective population sizes of 25 000 to 153 000 can be calculated for striped dolphins (original estimates of nucleotide diversity given in Garcia-Martinez *et al.*, 1995).

Considering that $N_{e(f)}$ represents the size of an ideal population that would display the same genetic diversity as that found in the study population, the effective population size estimated for bottlenose dolphins suggests one or more of the following has occurred: 1) the population was considerably larger, in the recent past, than the current census population, 2) east coast inshore bottlenose dolphin mtDNA was characterised by rapid radiation of haplotypes from a single founder or a few related founders or 3) repeated population bottlenecks and subsequent radiation of haplotypes maintained a constant low level of genetic variation. Data in Chapter 4 will be used to speculate which of these is more likely to have occurred.

3.4.4.2 Temporal effective population size (KwaZulu-Natal)

Knowledge of the approximate age of individuals (Cockcroft, unpublished data), enabled a division of the net-caught sample into two groups representing effectively two, albeit artificially defined, generations. Allele frequencies obtained for the polymorphic microsatellite loci were used to determine the short term effective population size of the KZN subpopulation. Using the temporal approach an effective population size of 31 animals was calculated for KZN, implying that the level of variation observed in the population over the two defined generations is similar to the variation one would expect to find in a population of fewer than 40 animals. Ideally, samples should be temporally discrete and should cover several generations. This is not always possible with long-lived cetacean species (Waples, 1991) and, given the short term over which bottlenose dolphins were sampled for this study, it would not have been possible to sample temporally discrete generations. Thus, this figure would almost certainly represent an under-estimate of N_e (Waples, 1991), as the true effects of genetic drift would be obscured by the effects of sampling within a single generation.

Several additional factors may bias the estimate. If the population is demographically unstable then the standardised variance of allele frequency change (F) (see section 3.2.4.4) will be biased upwards and N_e will be under-estimated (Waples, 1989). Capture data indicate that mature females and calves comprise the majority of the captures in shark nets (Cockcroft and Ross, 1991). This may have introduced a demographic bias to the KZN population over the 40 years of shark net installation. Furthermore, a greater likelihood of female and calf capture would likely bias a sample collected from the shark nets. Further bias may be introduced by subdivision within the sample. Although the possibility of bottlenose dolphins showing fidelity to restricted home ranges exits, significant subdivision is not evident within KZN (see Chapter 5). The primary factor biasing the estimation of temporal N_e is the absence of alleles at intermediate frequency (Waples, 1989). This would occur for loci with a single allele at high frequency and the remaining alleles at low frequency (eg. locus 199/200, Fig. 3.1). However, unless the estimate covers several generations, the bias would be small (Waples, 1989). As *t* represented only two generations, this factor would not be expected to affect N_e in this study.

Despite the likely under-estimation, N_e calculated from allele frequency variance is substantially smaller than both the demographic N_e and the census population (N). Demographic estimates of N_e vary widely among species, but are typically in the range 0.2 to 0.5N (Crow and Kimura, 1970; Mace and Lande, 1991). Using this range implies a demographic effective population of between 180 and 450 bottlenose dolphins on the KZN coast. Clearly, the demographic N_e and genetic N_e differ by up to an order of magnitude.

As a caveat regarding the use of effective population size determined from genetic data, it is important to consider that violations of the assumptions will bias estimates, often considerably. However, its usefulness lies as a relative rather than an absolute measurement (Hoelzel and Dover, 1991b) and its importance as a parameter in biological conservation is such that even an estimate that differs from the true value by as much as an order of magnitude can provide valuable biological information (Nei and Tajima, 1981). Thus, despite inherent difficulties in using the temporal method to estimate N_e from genetic data, the implication is that the effective size of the KZN bottlenose dolphin population is low. More important is the implication that the current level of genetic variation is similar to that of a population which is considerably smaller than the demographic N_e.

Table 3.6 Mitochondrial DNA nucleotide diversity and sequence divergence estimates for a range of marine mammal species, based on mtDNA control region sequence data.

	Nucleotide diversity (%)	Sequence divergence (%)	References
Pinnipeds			
California sea lions (*Zalophus californianus)*			Maldonado *et al.* (1995)
Pacific coast	0.41-1.71	1.1-1.4	
Gulf of California	1.02		
Pacific coast and Gulf of California		4-5.3	
Harbour seal (*Phoca vitulina*)			Stanley *et al.* (1996)
Atlantic Ocean		0.75	
Pacific Ocean		1.19	
Atlantic and Pacific Oceans		3.28	
Pacific harbour seal (*Phoca vitulina richardsi*)			Lamont *et al.* (1996)
Atlantic Ocean		0.75	
East Atlantic		0.23	
Pacific Ocean		1.19	
US Pacific coast		0.29	
Atlantic and Pacific Oceans		3.28	
Odontocetes			
Killer whale (*Orcinus orca*)			Hoelzel and Dover (1991a)
Resident sympatric populations		0.22	
Resident and transient communities		0.98	
Striped dolphin (*Stenella coeruleoalba*)	0.23	0.15-0.92	Garcia-Martinez *et al.* (1995)
Common dolphin (*Delphinus delphis*)			Rosel *et al.* (1994)
Short beak (California and ETP)	0.18	0.02	
Harbour porpoise (*Phocoena phocoena*)	1.5		Rosel *et al.* (1995a)
North Sea and Celtic Sea	0.81		Walton (1997)
Pacific	0.90		Rosel *et al.* (1995b)
Atlantic	0.89		

Table 3.6 cont'd

Burmeister's porpoise (*Phocoena spinipinnis*)	0.39		Rosel *et al.* (1995a)
Dall's porpoise (*Phocoenoides dalli*)	1.4		
Vaquita (*Phocoena sinus*)	0.0		Rosel and Rojas-Bracho, 1993)
Narwhal (*Monodon monoceros*)			Palsbøll *et al.* (1994)
East and West Greenland and Eastern Canadian archipelago	0.15		
Mysticetes			
Humpback whales (*Megaptera novaeangliae*)			Baker *et al.* (1993)
Individuals representing three ocean basins	2.57		
Minke whale (*Balaenoptera acutorostrata*)			
Antarctic	1.59		Bakke *et al.* (1996)
North Alantic	0.64 0.72		Bakke *et al.* (1996) Palsbøll *et al.* (1995)

Table 3.7 Microsatellite DNA genetic variability measures in a variety of large and small mammals, reptiles and insects. Mean H = average heterozygosity, mean A = average number of alleles per locus, mean A_p = average number of alleles per polymorphic locus.

Species	Mean H	Mean A (A_p)	Reference
Pilot whales (*Globicephala melas*)	-	6.0[*]	Amos *et al.* (1993)
Grey seals (*Halichoerus grypus*)	0.745	8.0	Allen *et al.* (1995)
Ethiopian wolf (*Canis simensis*)	0.205	2.4 (2.4)	Gotelli *et al.* (1994)
Domestic cat (*Felis catus*)	0.770	6.3	Menotti-Raymond and O'Brien (1995)
Puma (*Puma concolor*)	0.610	4.9	Menotti-Raymond and O'Brien (1995)
Lion (*Panthera leo*)	0.660	4.3	Menotti-Raymond and O'Brien (1995)
Cheetah (*Acinonyx jubatus*)	0.390	3.4 (4.4)	Menotti-Raymond and O'Brien (1995)
Arctic grizzly bears (*Ursus arctos*)	0.750	7.6	Craighead *et al.* (1995)
White-tailed deer (*Odocoileus virginianus*)	0.620	7.6	DeWoody *et al.* (1995)
Koala (*Phascolarctos cinereus*)	0.540	6.5	Houlden *et al.* (1996)
Northern hairynosed wombat (*Lasiorhinus krefftii*)	0.260	1.9 (3.1)	Taylor *et al.* (1994)
Southern hairynosed wombat (*Lasiorhinus latifrons*)	0.610	5.2 (5.3)	Taylor *et al.* (1994)
Green turtle (*Chelonia mydas*)	0.690	15 (18)	FitzSimmons *et al.* (1995)
Loggerhead turtle (*Caretta caretta*)	0.530	7.0 (10)	FitzSimmons *et al.* (1995)
Hawksbill turtle (*Eretmochelys imbricata*)	0.570	8.7	FitzSimmons *et al.* (1995)
Pacific ridley turtle (*Lepidochelys olivacea*)	0.320	3.2 (4.3)	FitzSimmons *et al.* (1995)
Flatback turtle (*Natator depressus*)	0.320	3.7	FitzSimmons *et al.* (1995)
Leatherback turtle (*Dermochelys coriacea*)	0.400	5.2 (6)	FitzSimmons *et al.* (1995)
Timber rattlesnake (*Crotalus horridus*)	0.380	4.8	Villarreal *et al.* (1996)
Social wasp (*Polistes annularis*)	0.620	4.8	Hughes and Queller (1993)
Humpback whale (*Megaptera novaeangliae*)	0.720	12.75	Valsecchi *et al.* (1997)
Humpback dolphin (*Sousa chinensis*)[§]	0.460	2.0 (2.8)	This study
Humpback dolphin (*Sousa chinensis*)[+]	0.240	1.6 (2.6)	This study
Bottlenose dolphin (Tursiops truncatus)[+]	0.290	1.9 (2.5)	This study

[*] Average for five loci scored for pilot whales. The sixth locus produced 54 alleles.

[+] South African population.

[§] Hong Kong population.

3.5 CONCLUSIONS

Four molecular systems have indicated low genetic variation in South African bottlenose dolphins. The current level of variation may be an historical characteristic of this population, caused by events such as a sudden numerical decline or a recent founder event from which the population arose (see Chapter 4). Nonetheless, bottlenose dolphins off the KZN coast have been subject to depletion pressure by shark nets for approximately four generations. Continued exploitation and depletion of an already small population will further reduce genetic variation and with it, the inclusive fitness of the population. The consequence of this would be reduced resilience of the population to stochastic influences and reduced ability to recover from the decline. Although the results from this study require confirmation from additional analysis of unsampled regions, the indication is that genetic variation in KZN and East Cape bottlenose dolphins is low and that measures should be taken to prevent further depletion off the KZN coast (see Chapter 8).

CHAPTER 4

MITOCHONDRIAL DNA PHYLOGENY AND EVOLUTIONARY HISTORY OF S.E. AFRICAN BOTTLENOSE DOLPHINS

4.1 INTRODUCTION

Genetic variability measures have suggested that KZN and East Cape bottlenose dolphins display low levels of diversity. For the purpose of conservation and management of the species off KZN, it is necessary to determine whether this variation is a product of current and recent exploitation or whether it can be attributed to events in the history of the population. Furthermore, recommendations for managing and conserving two proposed stocks of bottlenose dolphin (Peddemors, 1995) require consideration of their phylogenetic distinctiveness and genetic distance before management priority is assigned (Moritz, 1994).

4.1.1 Mitochondrial DNA divergence

Different mutation rates and mechanisms govern the evolution of different regions of DNA and it has been proposed that management decisions be based on corroborating genetic data from both nuclear and extranuclear DNA (Vogler *et al.*, 1993; Moritz, 1994). However, the haploid state renders mtDNA more susceptible than nuclear DNA to the effects of genetic drift, population bottlenecks and subdivision (Barton and Jones, 1983; Harrison, 1989; Amos and Hoelzel, 1992). Thus, where genetic variation exists, it is more easily detected in the mtDNA than in most nuclear genes (Moritz, 1994).

Before conservation decisions can be made regarding bottlenose dolphins in South Africa, the level of intra- and interpopulation divergence must be assessed. Furthermore, knowledge of historical divergence can play a critical role in interpreting current patterns of variation. The control region of the mtDNA is ideal in this regard as divergence can be resolved over time scales of 10 000s to 100 000s of years (Billington and Hebert, 1991; Palumbi *et al.*, 1991), permitting inferences regarding the effects of historical ecology and stochasticity on current genetic variation (Palumbi *et al.*, op cit).

4.1.2 Phylogeny reconstruction

Phylogeny is inferred by assuming the inheritance and mutation of ancestral genetic characteristics which define the evolutionary history of a lineage (Swofford and Olsen, 1991). Two important aspects of intraspecific mtDNA variability can be assessed using estimates of phylogeny. They are the patterns and degrees of differentiation among haplotypes and the geographic distributions of phylogenetic groupings (Avise, 1989a). Thus, a determination of the phylogeny of mtDNA haplotypes within a population or species provides an indication of both the historical evolution of, and current genetic divisions within that population or species.

4.1.3 Measures of phylogeny estimation

Parsimony analysis comprises a group of related methods with the common goal of minimising the number of evolutionary steps required to explain a given set of data (Swofford and Olsen, 1991). A step may comprise a base substitution in nucleotide sequence data or a gain or loss event in restriction site data. The parsimony criterion makes use of the smallest number of steps required for the trees examined and finally chooses the tree requiring the fewest changes (Felsenstein, 1988).

Constructing phylogenies using the maximum likelihood method, concerns estimating the likelihood that the observed sequences will occur under a given evolutionary model (Felsenstein, 1981, 1988), whereas distance matrix methods estimate corrected distances which are then used to generate trees via neighbour-joining (Saitou and Nei, 1987), UPGMA or minimum evolution reconstruction methods. Whereas minimum evolution makes use of an explicit optimality criterion (Swofford and Olsen, 1991), the neighbour-joining method makes use of an algorithm which minimises the sum of branch lengths at each stage of clustering of taxa, working inwards from a starlike tree (Saitou and Nei, 1987). Although it may not resolve the shortest tree, it is efficient at presenting the correct topology (op cit.).

4.1.4 Objectives

Sequence data from the first 400 bases of the mtDNA control region are used to trace the divergence and intraspecific phylogenetic relationships of inshore bottlenose dolphins on the South African coast. Genetic data are also discussed in light of the geological history of the coast, to investigate the influence of environmental events on current phylogeny and variability. The following analyses were conducted:

1) estimation of the mtDNA divergence among South African bottlenose dolphins, and
2) reconstruction of the phylogeny of inshore bottlenose dolphin mtDNA haplotypes.

4.2 MATERIALS AND METHODS

4.2.1 Sample collection

Samples were collected as described in sections 2.2.1.1 and 2.2.2.1. Two offshore bottlenose dolphin sequences were used in phylogenetic reconstruction. One sample originated off the South African west coast and the second sequence was donated by Dr Barbara Curry (see section 2.2.2.1 for further detail).

4.2.2 DNA analysis

DNA extraction, amplification, sequencing and electrophoresis were conducted as described in sections 2.3 to 2.5.

4.2.3 Data analysis

4.2.3.1 DNA divergence

Sequence divergence was calculated as the number of net nucleotide substitutions between two populations (Nei, 1987). This method accounts for the effect of sequence polymorphism within populations before estimating the sequence divergence between them. The average number of nucleotide substitutions between any pair of haplotypes in a population was estimated as $d_x = n_x/n_x-1\ [\Sigma_{ij}\ (x_i x_j d_{ij})]$, where n_x is the number of haplotypes sampled, x_i and x_j are the sample frequencies for the ith and jth haplotype and d_{ij} is the number of nucleotide subsitutions per site, estimated by the Kimura 2-parameter method (Kimura, 1980) with a gamma correction ($\alpha = 0.5$) to account for among-site rate variation (Jin and Nei, 1991). Thereafter, the average number of nucleotide substitutions between any two haplotypes from the two populations was estimated by $d_{xy} = \Sigma_{ij}(x_i\ y_j\ d_{ij})$, where d_{ij} is the number of nucleotide substitutions between the ith haplotype from population X and the jth haplotype from population Y. Finally, the net substitutions between the two populations was estimated by $d_a = d_{xy} - [(d_x + d_y) / 2]$ (Nei, 1987).

4.2.3.2 Phylogeny reconstruction

Phylogenetic analyses were based on three different methods. Parsimony analyses were performed using PAUP 3.1.1 (Swofford, 1993) and MEGA (Kumar *et al.*, 1993). Branch and bound searches were performed to identify all minimum-length trees. Initially, transitions and transversions were weighted equally; however, the empirical Ti/Tv ratio was also used to weight transversions over transitions to more accurately approximate the mode of nucleotide evolution. The *g1* statistic was used to determine the significance of the phylogenetic signal provided by the data (Hillis and Huelsenbeck, 1992).

A neighbour-joining tree was constructed (Phylip 3.5, Felsenstein, 1993) using the genetic distances determined above (Kimura, 1980). The statistical confidence of each node was determined by bootstrap analysis with 500 iterations (Felsenstein, 1985). In addition, MEGA (Kumar *et al.*, 1993) was used to construct a neighbour-joining tree using the simplified version of the minimum evolution method (Saitou and Nei, 1987). The statistical confidence was tested using 1000 bootstrap resamplings.

A maximum likelihood tree was constructed by the DNAML program in the PHYLIP (Phylogeny Inference Package) version 3.5 software package (Felsenstein, 1993), using empirical base frequencies. Global re-arrangement and the jumble option were used to improve the probability of finding the tree with the greatest likelihood.

4.3 RESULTS

4.3.1 DNA divergence

Genetic distance ranged from 0.25% to 3.02% (1.04% excluding haplotype 5) with a mean of 0.98% (0.63% excluding haplotype 5) among east coast haplotypes (Table 4.1). Sequence divergence (Nei, 1987) of 0.104% was estimated among haplotypes within KZN and 0.080% among haplotypes in the East Cape sample. After compensating for sequence polymorphism within either region, divergence between KZN and the East Cape was estimated at 0.121%. This value increased to 3.10% between east coast haplotypes and the west coast offshore sample (haplotype 12) and 3.97% between east coast haplotypes and the US Atlantic offshore sample (haplotype 13).

A somewhat divergent sequence was obtained for a single sample taken off KZN. The sequence divergence between haplotype 5 and the remaining nine east coast haplotypes (2.46 %) was substantially larger than the average divergence among the nine haplotypes (0.121 %). Data sheets completed during necropsy identified the animal, captured in a shark net, as a bottlenose dolphin calf. The rostral teeth had barely erupted and there were no mandibular teeth (P.E.M. data sheets), indicating that the animal was a neonate. Originally believed to be an inshore bottlenose dolphin, by virtue of its capture in a shark net, the animal (146 cm) exceeded the average length of inshore neonates (range 86-115 cm, mean 103 ± 7.6 cm, Cockcroft and Ross, 1989), suggesting that it was probably a larger, offshore bottlenose dolphin.

4.3.2 Phylogenetic analysis

Of the 400 bases of the control region sequenced, 29 were variable and although only 12 were parsimony informative, a *g1* of -1.327 suggested the sequence data contained significant phylogenetic signal (Hillis and Huelsenbeck, 1992). A branch and bound search using PAUP version 3.1.1 (Swofford, 1993) generated 11 trees of 36 steps, with a confidence index (CI) of 0.806. A strict consensus of these trees, shown in Figure 4.1, presents the coastal haplotypes as a monophyletic clade with the east coast offshore haplotype (haplotype 5) placed basally to these. With only 12 informative characters in the data set, this tree displays short branch lengths, indicating very little variation among haplotypes.

The neighbour-joining tree divides the KZN unique haplotypes into two clades, one of which is combined with the East Cape haplotypes to form a larger clade in 32 % of the resamplings. Haplotype 4, represented by KZN samples by an 81 % majority, is placed in a clade with the East Cape haplotypes in 27 % of the resamplings generated with the neighbour-joining algorithm in PHYLIP 3.5 (Felsenstein, 1993; Fig. 4.2). A similar topology was generated using the neighbour-joining option in MEGA (Kumar *et al.*, 1993). Although the topologies of trees constructed with slightly different algorithms are similar, low bootstrap values at the nodes do not place a high degree of confidence in the branching order of the trees. This suggests that insufficient differences among haplotypes have generated small genetic distances, providing insufficient information to either support or collapse certain branches.

The maximum likelihood tree (Fig. 4.3) was determined from 9359 trees under the Kimura 2-parameter model (Kimura, 1980), using the empirical Ti/Tv of 14:1. However, given that this value is based on a single transversion, it is probably not accurate. This method presents the KZN haplotypes as a clade within an East

Cape clade. Nonetheless, the assignment of the two major haplotypes, 2 and 4, to separate sister clades comprising unique haplotypes from the East Cape and KZN, respectively, corresponds with the relative proportion of those haplotypes present in either region.

Table 4.1. Nucleotide variation in 13 bottlenose dolphin mitochondrial haplotypes. Genetic distance (in percent) is expressed as gamma distances (α = 0.5) calculated using the Kimura 2-parameter model (Kimura, 1980) (above diagonal). The number of nucleotide substitutions is partitioned into transitions / transversions (below diagonal). Haplotypes 12 and 13 represent the two outgroups (see section 2.2.2.1). Haplotype numbers correspond with those in Table 3.1.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1		0.51	0.25	0.25	1.04	0.77	0.77	0.77	0.51	0.51	0.77	3.29	4.18
2	2/0		0.25	0.25	2.72	0.77	0.77	0.77	0.51	0.51	0.77	3.29	4.18
3	1/0	1/0		0.51	2.42	1.04	1.04	1.04	0.77	0.77	1.04	3.59	4.50
4	1/0	1/0	2/0		2.42	0.51	0.51	0.51	0.25	0.25	0.51	2.99	3.87
5	8/0	10/0	9/0	9/0		3.01	2.42	3.01	2.72	2.72	3.01	3.29	5.47
6	3/0	3/0	4/0	2/0	11/0		1.04	0.51	0.77	0.25	1.04	3.59	3.87
7	3/0	3/0	4/0	2/0	9/0	4/0		1.04	0.25	0.77	0.51	2.99	4.5
8	3/0	3/0	4/0	2/0	11/0	2/0	4/0		0.77	0.25	1.04	3.59	3.87
9	2/0	2/0	3/0	1/0	10/0	3/0	1/0	3/0		0.51	0.25	2.7	4.18
10	2/0	2/0	3/0	1/0	10/0	1/0	3/0	1/0	2/0		0.77	3.29	3.57
11	3/0	3/0	4/0	2/0	11/0	4/0	2/0	4/0	1/0	3/0		2.99	4.18
12	11/1	11/1	12/1	10/1	11/1	12/1	10/1	12/1	9/1	11/1	10/1		4.16
13	13/2	13/2	14/2	12/2	17/2	12/2	14/2	12/2	13/2	11/2	13/2	12/3	

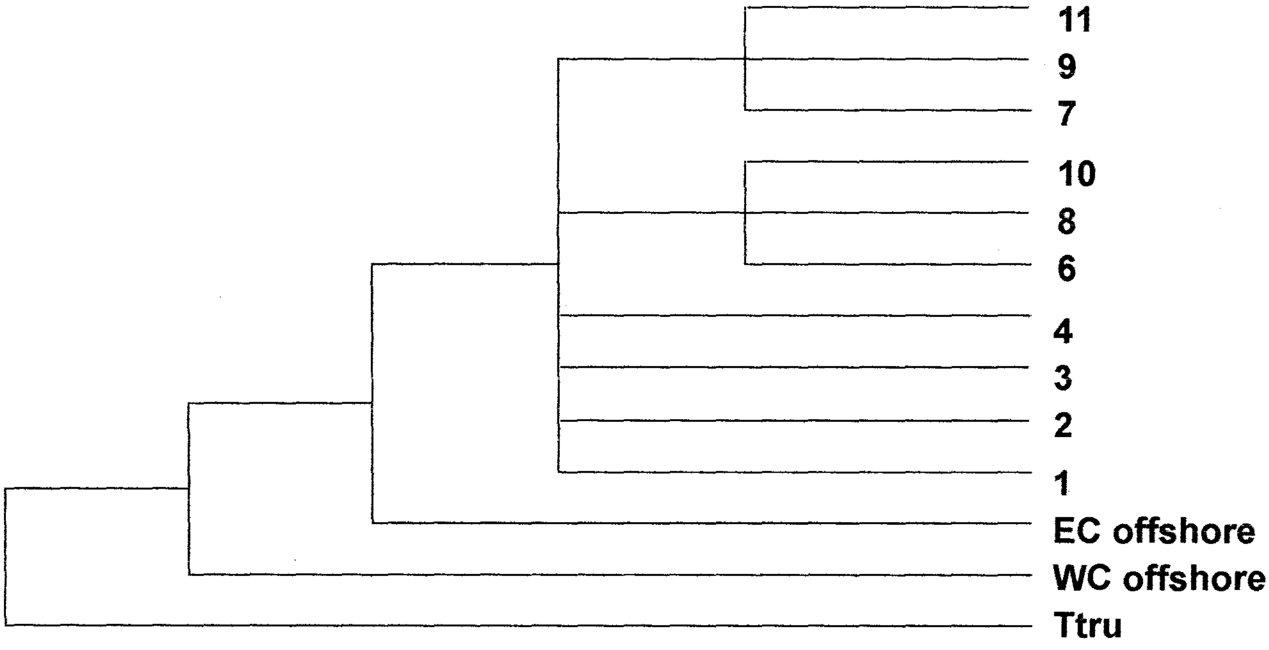


Figure 4.1 A strict consensus of 11 maximum parsimony trees retained from a branch-and-bound search using PAUP 3.1.1. All characters were specified as unordered and assigned equal weight. The total length of the tree is 36 steps and the CI is 0.806. The *g1* statistic, reflecting tree length distribution is -1.327. Haplotype names and numbers correspond with those in Table 3.1.

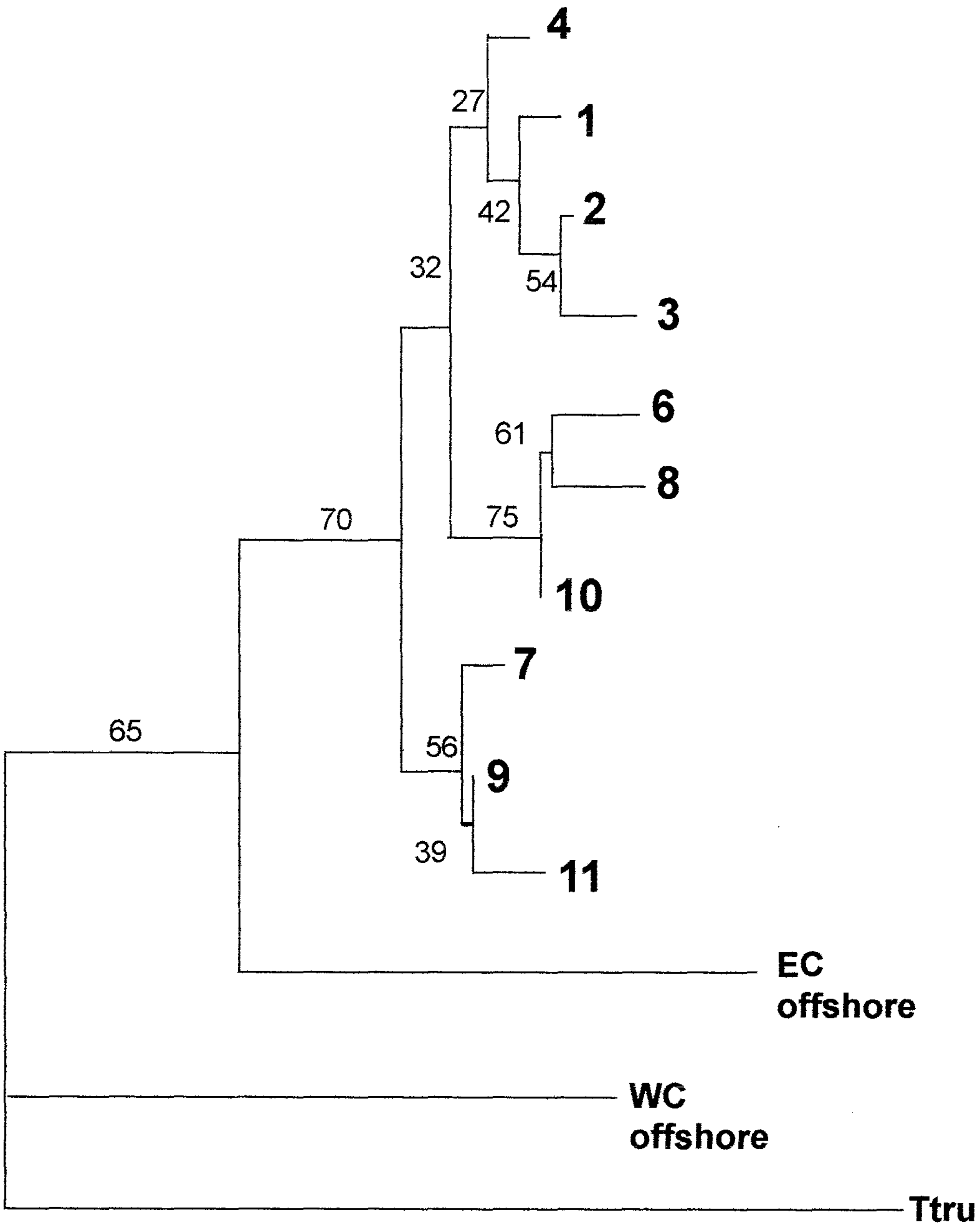


Figure 4.2 A strict consensus tree generated from 500 bootstrap replications using the neighbour-joining algorithm in PHYLIP 3.5. Distances were estimated using the Kimura 2-parameter model with a gamma correction with a shape parameter of 0.5. Numbers at the nodes represent bootstrap values as percentages. The branches are scaled in terms of distance units. Haplotype names and numbers correspond with those in Table 3.1.

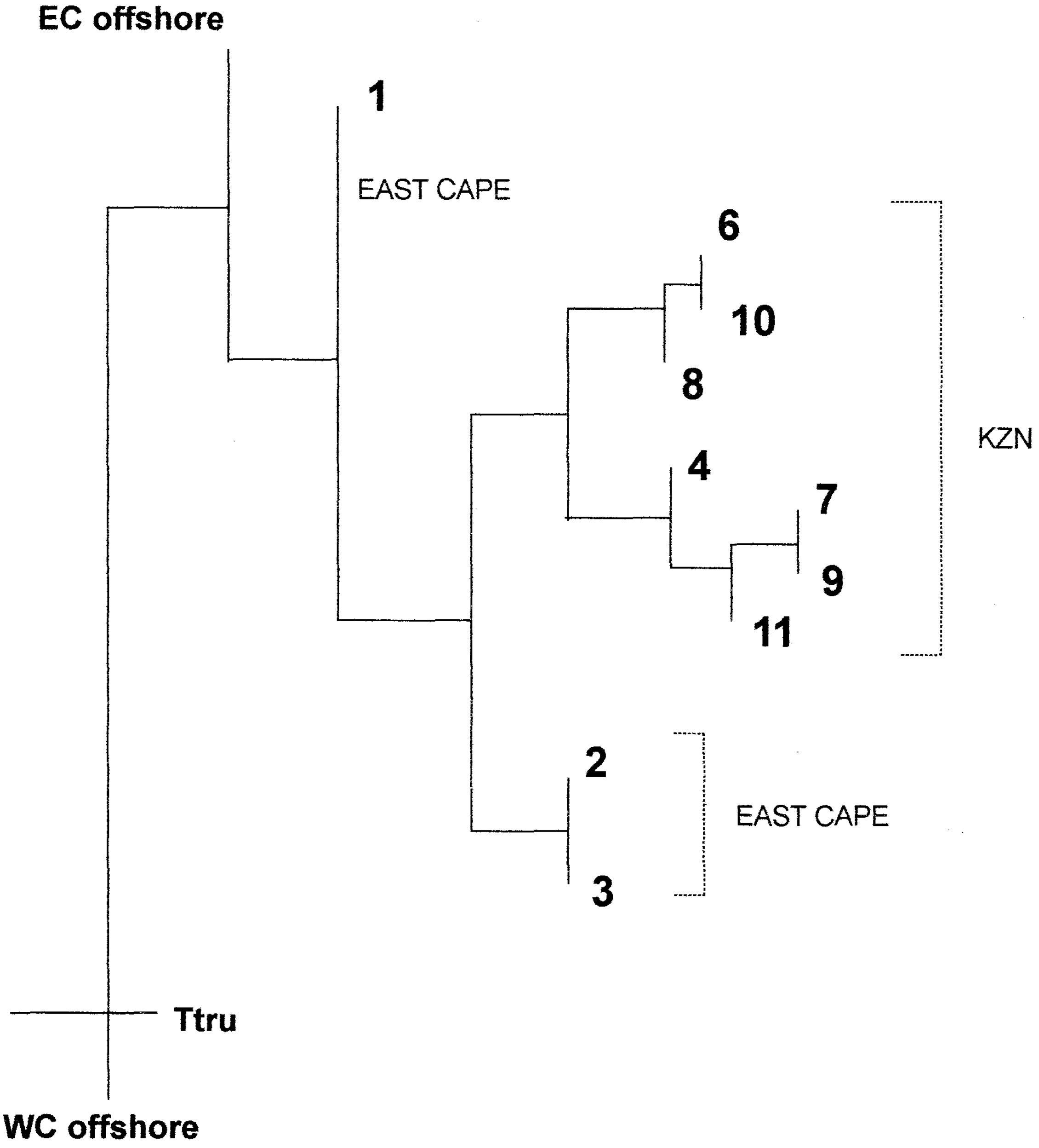


Figure 4.3 Maximum likelihood of 9359 trees obtained using PHYLIP 3.5. Branch lengths are scaled in terms of number of substitutions per site. Log likelihood is -743.08. Haplotype names and numbers correspond with those in Table 3.1.

4.4 DISCUSSION

4.4.1 DNA divergence

4.4.1.1 Coastal haplotypes

The average interpopulation sequence divergence of bottlenose dolphin mitochondrial haplotypes (0.121 %) falls within the range reported for a variety of species reviewed in Vogler and DeSalle (1994). Moreover, it falls within the large range of intraspecific divergence values described for many marine mammal species (0.02 % to 5.4 %; Table 3.6) and is similar to values given for other coastal delphinids. Hoelzel and Dover (1991a) reported 0.22 % sequence divergence between partially sympatric resident killer whale (*Orcinus orca*) populations, while differences of 0.15 % were reported between striped dolphin haplotypes from the Mediterranean coast (Garcia-Martinez *et al.*, 1995). Bottlenose dolphins sampled from the US Pacific coast differed by between 0.08 and 0.58 %, while specimens from the Gulf of Mexico (Dowling and Brown, 1993) displayed a similar level of divergence to coastal bottlenose dolphins in this study (Table 3.6).

4.4.1.2 Support for inshore and offshore forms

Several researchers have proposed a distinction between coastal and offshore forms of the bottlenose dolphin (Ross, 1977; Duffield *et al.*, 1983; Ross and Cockcroft, 1990; Dowling and Brown, 1993; Curry *et al.*, 1994, 1995). Morphological differences between the two off south east Africa include variations in body length, tooth counts and skull and vertebral features (Ross, 1977; Ross and Cockcroft, 1990).

The first suggestion that inshore and offshore bottlenose dolphins may differ genetically, came from studies of haematology parameters which distinguished the two forms in the Atlantic and Pacific Oceans (Duffield *et al.*, 1983). Subsequently, restriction analysis of the mtDNA revealed a highly divergent haplotype among coastal bottlenose dolphins sampled in the Gulf of Mexico (Dowling and Brown, 1993). It was proposed that the two animals scored for that haplotype may have originated from a distinct offshore stock (op cit). Current thinking (Curry *et al.*, 1995) proposes a revision of the taxonomy of the genus *Tursiops*, with particular reference to differences between inshore and offshore forms. Recently, D-loop sequence data suggested that the inshore and offshore forms of *Tursiops* represent different species (Curry *et al.*, 1995), while cytochrome *b* sequence data placed the inshore (aduncus) form in a clade with *Stenella* and *Delphinus* species, suggesting that *Tursiops* represents a paraphyletic genus (Le Duc, in preparation). Both studies included sequence data from South African inshore bottlenose dolphins.

The single divergent haplotype found among inshore dolphins taken from KZN shark nets, provides the first genetic support for divergence between inshore and offshore forms off the east coast of South Africa. Furthermore, agreement among the three phylogenetic methods on the placement of the east coast offshore haplotype (haplotype 5) basal to inshore haplotypes is further evidence for distinct differences between the two forms. Larger divergences and greater branch lengths were found between east coast inshore and west coast offshore haplotypes (> 3 %) and between the South African east coast and the US Atlantic offshore haplotype (4 - 5.5 %), supporting the view of Curry *et al.* (1995) that globally, inshore and offshore bottlenose dolphins may comprise separate species.

4.4.2 Mitochondrial DNA phylogeny

4.4.2.1 Estimation of phylogeny

A neatly bifurcating phylogeny corresponding to the geographical distribution of haplotypes may have been expected for KZN and East Cape bottlenose dolphins. Although division of the two regions is weakly suggested (eg. Figs. 4.2 and 4.3), few differences among the haplotypes produced phylogenies with short branch lengths and low bootstrap support. These results support the earlier suggestion (section 3.4.4.1) that the population has undergone limited but rapid radiation following either the establishment of a small, maternally-related founder population or a bottleneck survived by only a few haplotypes.

4.4.2.2 Evolutionary history of South African inshore bottlenose dolphins

All genetic systems studied to date (Goodwin *et al.*, 1996; Chapter 3, this study) suggest that South African coastal bottlenose dolphins are characterised by low genetic variability. This is supported by short internal branch lengths and low bootstrap values in the mtDNA phylogeny. The suggestion from these data is that the population was either recently founded or experienced a severe bottleneck during its recent evolutionary history.

The time since divergence of two haplotypes can be estimated using the relationship $r = d/2T$ (Kimura, 1980; Wilson *et al.*, 1985; Hoelzel and Dover, 1991b), given the average sequence divergence and the relevant rate of evolution. Given an average divergence of 0.12 % within inshore bottlenose dolphins and a divergence rate of 0.5 - 1.0 % per million years (MY) for the cetacean D-loop (Hoelzel *et al.*, 1991b; Baker *et al.*, 1993), the time since homogeneity of South African bottlenose dolphin mtDNA can be calculated at between 78 000 and 120 000 years before the present (BP).

Historical evidence suggests that inshore dolphins were more likely to have experienced a severe bottleneck in their recent evolutionary history. Current levels of mtDNA diversity suggest that the bottleneck was followed by a recovery period, during which limited, but rapid radiation of mtDNA haplotypes may have occurred. If mtDNA diversity had declined to a single haplotype, current levels of diversity would date the bottleneck to 78 000 - 120 000 years BP (sensu Menotti-Raymond and O'Brien, 1993). This corresponds approximately to the period from the onset to the middle of the last glacial age (Deacon and Lancaster, 1988). The Pleistocene era was characterised by frequent and rapid changes in the world climate (CLIMAP, 1976), during which time glaciation exerted a profound effect on coastal waters, primarily with regards to declines in temperature and sea level (CLIMAP, 1976; Deacon and Lancaster, 1988). Population bottlenecks associated with the Pleistocene glaciation appear to have impacted severely on many temperate fish populations. Billington and Hebert (1991) report several cases of the effects of bottlenecks on the extent of haplotype diversity and divergence in species found in both the northern and southern hemispheres during that period.

Estimations of sea level during the last glacial low stand range from 85 to 150 m below present levels (CLIMAP, 1976; Deacon and Lancaster, 1988). More appropriately, it declined to -110 m and -130 m, respectively, on the west and south coasts of South Africa (Deacon and Lancaster, 1988). The shallow continental shelf is extremely narrow along the south eastern coast and the shelf break currently occurs at a depth of approximately 100 m (Heydorn *et al.*, 1978). Clearly, a decline in sea level of over 100 m would have resulted in the disappearance of the shelf. The absence of a shallow coastal habitat would almost certainly have prohibited the survival of inshore dolphins.

Furthermore, during the last glacial maximum, surface water temperatures declined by as much as 4°C during the austral winter (CLIMAP, 1976). During this period, it appears that the warm Agulhas Current, which presently passes the south east African coast (see Chapters 5 and 7), did not feature prominently in the circulation of Indian Ocean waters and the warm Mozambique Current, which presently feeds the Agulhas Current, may have turned east to parallel the West Wind Drift (CLIMAP, 1976). Consequently, a lack of southward-moving tropical waters would have thermally isolated the southern African coast. As a temperate species, bottlenose dolphins may have withstood temperatures which were lower than contemporary values. However, regular, large fluctuations would probably have contributed to a decline in survival.

4.5 CONCLUSIONS

Consideration of the genetic data obtained in this study and the geological history of the region, suggests that climatic and environmental factors may have initiated a rapid decline in the coastal bottlenose dolphin population during the last glacial age. Recovery appears to have been characterised by a rapid radiation of haplotypes as suggested by the current level of variation in the region of mtDNA studied.

Although the recent exploitation and suspected decline in number of bottlenose dolphins is probably not responsible for the current low genetic variation, the results from this study should be regarded as a caveat against continued exploitation of the KZN population. At the current level of diversity, continued depletion may not be sustainable and post-exploitation recovery of genetic variation will be hindered if the remaining population is too small (Bonnell and Selander, 1974; Hoelzel *et al.*, 1993). Recruitment from areas outside of the netted region may offset the depletion within it (see Chapter 5). However, if all measures of genetic variability are low in bottlenose dolphins along the entire length of the south east coast, then recruitment from adjacent regions will only serve to slow the depletion of numbers, without the infusion of additional diversity.

CHAPTER 5

GENETIC STRUCTURE AND GENE FLOW IN S.E. AFRICAN BOTTLENOSE DOLPHINS

5.1 INTRODUCTION

5.1.1 Population differentiation in the marine environment

Often geographically separate populations may be genetically similar in marine organisms. This is a result of the potential for both active and passive movement over long distances in the marine environment. Patterns of differentiation among terrestrial organisms can be understood as consequences of interactions between known historical, topographic or environmental factors. In contrast, life history characteristics and social factors may be the primary influence on the degree of genetic differentiation among populations of marine organisms rather than barriers to dispersal or geographic distance.

5.1.2 Estimation of population structure

The genetic structure of populations is studied by assessing the departure of allele frequencies observed in study populations from frequencies expected in panmictic populations (Excoffier *et al.*, 1992). Wright (1951) introduced the parameters F_{IT}, F_{IS} and F_{ST} as a means of describing population structure. These measure, respectively, the reduction in heterozygosity of an individual relative to the whole population, relative to a subpopulation within the whole population and the reduction of heterozygosity of a subpopulation relative to the whole population (Hartl, 1988). Several similar estimation procedures have been used in polymorphic systems (Cockerham, 1969, 1973; Nei, 1977). Weir and Cockerham (1984) presented a means of estimating F-statistics without making assumptions about sample size and the number of populations sampled, thus facilitating the analysis of small data sets and the comparison of results from independent studies.

Recently, Slatkin (1995) introduced the R_{ST} statistic for the analysis of microsatellite loci. An analogue of F_{ST}, R_{ST} is estimated under a model of stepwise mutation which may be a more appropriate model for the evolution of microsatellite alleles than the infinite alleles model (Edwards *et al.*, 1992; Valdes *et al.*, 1993; Goldstein *et al.*, 1995 a and b). As with F_{ST} (Wright, 1951) or θ (Weir and Cockerham, 1984), R_{ST} represents an among-population component of variance and can be estimated for several loci (Slatkin, 1995).

A useful means of studying molecular variation at the level of mtDNA is the analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). In this procedure, divergence among DNA haplotypes, in the form of squared-distances, is incorporated into an analysis of variance format. The analysis of molecular variance (AMOVA) examines a hierarchy of variance components and derives F-statistic analogues (designated ϕ-statistics) describing the correlation between genetic variance and geographical subdivision. An important advantage over analogous models is that the AMOVA is not critically dependent on the fulfilment of specific evolutionary assumptions. This makes it invaluable as a generic estimator of population differentiation, sufficiently flexible for the analysis of molecular systems such as nucleotide sequences, allozymes and microsatellite DNA (Michalakis and Excoffier, 1996).

5.1.3 Gene flow

Knowledge of the extent of gene flow between populations is an important requisite to understanding evolution (Slatkin and Barton, 1989). Furthermore, the interplay between genetic differentiation and gene flow is what determines the level at which populations are managed and conserved. Gene flow is defined as the "change in gene frequency due to movement of gametes, individuals or groups of individuals from one place to another" (Slatkin, 1987). It functions to either restrict evolution by preventing adaptation to local conditions or encourage evolution by dispersing new gene combinations throughout a species' range (Slatkin, 1987).

Gene flow is monitored directly, by observing the dispersal of individuals or gametes (Slatkin, 1985), or indirectly, by assessing the spatial distributions of gene frequencies and DNA sequence differences to infer past gene flow (Slatkin, 1987). Several methods have been presented to infer gene flow from genetic data. Wright (1951) showed that for neutral alleles, $F_{ST} \sim 1/(1+4Nm)$ where N is the local popn size and *m* is the average rate of immigration in an island model of population structure. The relative strengths of gene flow and genetic drift can be compared with this combination of parameters and by inverting the formula, N*m* can be estimated from F_{ST}.

Takahata and Palumbi (1985) showed that, for extranuclear genomes, the relationship introduced by Wright (1951) can be modified to $G_{ST} = 1/(1+2Nm)$. Using an analogue of G_{ST}, such as ϕ_{ST} (Excoffier *et al.*, 1992), N*m* can be determined from mtDNA sequence information.

5.1.4 Population subdivision within and among KZN and East Cape bottlenose dolphins.

It has been suggested in Chapters 3 and 4, that dolphins in KZN and the East Cape display low genetic diversity, probably as a result of stochastic environmental events which occurred during the history of the species on the south east African coast. This places added importance on recommendations for the conservation and management of inshore dolphins along the KZN coast, in light of current exploitation in that region.

A determination of the structure and degree of differentiation within the bottlenose dolphin population has been the focus of both intensive field research and preliminary genetic analysis. Structure within the KZN group has been suggested from field and observational data accumulated over the last 15 years. Observations during aerial surveys along the KZN coast suggested regions of peak sighting represented 'preferred areas' occupied by bottlenose dolphins (Ross *et al.*, 1987, 1989; Cockcroft *et al.*, 1990b). These regions, approximately 37km in length, are suspected to be the home ranges within which groups of bottlenose dolphins reside. No direct evidence is available to indicate site fidelity or strict residence of bottlenose dolphin groups.

However, several other studies have pointed to some degree of differentiation within the KZN population: Cockcroft *et al.* (1989b) reported a significant difference in the concentration of organochlorines found in the blubber of bottlenose dolphins on the KZN north and south coasts. Peddemors (1995) noticed geographical and temporal differences in the intensity of ventral spotting of bottlenose dolphins caught in shark nets and although this feature could not be used reliably as an indicator of the population of origin, it suggested possible phenotypic differentiation along the coast. For at least 25 years, there have been anecdotal reports of bottlenose dolphins removing fish from the lines of commercial fishermen (Garratt, 1980; Ross *et al.*, 1987) in a single region off the KZN south coast. The activity appears to be limited to the animals in that area, despite the obvious advantages it may have for dolphins encountering commercial fishing lines on other parts of the coast, implying that some schools may be resident in certain localities along the coast. The first genetic study of KZN bottlenose dolphins investigating the variability in allozyme loci, failed to detect significant differentiation among 'preferred areas' in KZN (Goodwin *et al.*, 1996). Nevertheless, greater variability on the south coast and greater genetic distances between 'preferred areas' on the north coast, suggested a trend towards weak subdivision. However, the authors suggested that this may have resulted from the inclusion of 'migrant' dolphins in the south coast sample (op cit.).

It has been suggested that bottlenose dolphins in KZN and the East Cape comprise two separate stocks (Peddemors, 1995; Goodwin *et al.*, 1996), members of which meet annually on the KZN south coast. An annual influx of between 1900 and 2300 bottlenose dolphins occurs from regions south of KZN (Peddemors, 1995), behind the annual pilchard (*Sardinops sagax*) migration (Baird, 1971; Heydorn *et al.*, 1978). Only evident during the winter months, these large groups are suggested to be a migrant stock of bottlenose dolphins perhaps engaged in an extention of their range in response to a shift in prey abundance (Peddemors, 1995). These observations led to the proposal that dolphins on the KZN south coast comprised a 'resident' stock and a 'migrant' stock (op cit.). It was further suggested that these migrants represent an East Cape population which is distinct from that off KZN, although the extent of differentiation and the degree of breeding interaction between the two regions is unknown. Determination of whether dolphins from the two regions are, in fact, genetically different, is necessary before proposals are made to manage them as separate stocks.

5.1.5 Objectives

This chapter of the study aims at estimating the degree of genetic differentiation and gene flow among groups of bottlenose dolphins off the KZN coast as well as testing for differentiation between the KZN and East Cape regions. This was undertaken by:

1) determining the degree of differentiation from a portion of the mtDNA d-loop at several hierarchical levels using AMOVA (Excoffier *et al.*, 1992),

2) determining the degree of differentiation in microsatellite DNA at several hierarchical levels using R_{ST} (Slatkin, 1995) and AMOVA,

3) estimating the degree to which spatial differentiation accounts for total population diversity in microsatellite DNA, using F_{ST} (Weir and Cockerham, 1984),

5) inferring levels of gene flow from F-statistics, ϕ-statistics and R_{ST} using the relationships of Wright (1951), Takahata and Palumbi (1985) and Slatkin (1995).

5.2 MATERIALS AND METHODS

5.2.1 Sample collection

Samples were collected as described in sections 2.2.1.1 and 2.2.2.1.

5.2.2 DNA analysis

DNA extraction, amplification, sequencing and electrophoresis were conducted as described in sections 2.3 to 2.5. Microsatellite analysis was performed as described in section 2.6. Gender determination of biopsy and stranding samples was conducted as described in section 2.8.

5.2.3 Terminology and group designation

KwaZulu-Natal and the East Cape are the two major regions under investigation in this chapter. Both regions are further divided into subregions for analysis of molecular variance (AMOVA) among population within regions. The KZN coast is subdivided into the north and south coasts, with the division occurring at Durban. The coast is further divided into six 'preferred areas' (Fig. 2.1 inset) designated from sighting data and aerial surveys (Ross *et al.*, 1987; Cockcroft *et al.*, 1990b). The East Cape has not been divided into subregions for this analysis.

The term 'stock' is used in this chapter to distinguish between dolphins sampled in either of the major regions. In this context, it is based on significant molecular differentiation and makes no assumptions about the degree of morphological or geographical divergence (Dizon *et al.*, 1992) or the evolutionary significance (Moritz, 1994a) of either group.

5.2.4 Data analysis

5.2.4.1 Population structure

5.2.4.1.1 Estimation of population structure from mtDNA

A hierarchical approach analogous to an analysis of variance (Excoffier *et al.*, 1992) was used to determine the degree of subdivision within and among designated 'preferred areas' and the two regional stocks. A matrix of squared Euclidean distances was constructed for all pairwise comparisons of the 10 mitochondrial haplotypes resolved for South African inshore bottlenose dolphins. Three components of genetic variance were calculated: ϕ_{CT} represents the fraction of the total variation accounted for by variation among groups of populations, ϕ_{SC} measures the fraction of total variation accounted for by variation among populations within a group and ϕ_{ST} quantifies the extent to which population differentiation accounts for the variation in the total population. This parameter is analogous to Wright's (1951) F_{ST} and Takahata and Palumbi's (1985) G_{ST}. The significance of each variance estimate is determined by comparing the observed ϕ statistics with values generated under a null distribution, calculated by a large number of random permutations of the distance matrix (Excoffier *et al.*, 1992).

5.2.4.1.2 Estimation of population structure from microsatellite DNA

Variation in allele frequencies was used to calculate population heterogeneity in microsatellite DNA. The degree to which spatial differentiation accounts for total population diversity is expressed as the jack-knifed mean of F_{ST}, θ, calculated using the method of Weir and Cockerham (1984) with the BIOSYS-1 program (Swofford and Selander, 1981). This estimation procedure accounts for the effects of sampling a limited number of individuals within a limited number of subpopulations. A simple weighting procedure allows the combination of data over all alleles and loci (Weir and Cockerham, 1984).

Recently, it was shown that the AMOVA (Excoffier *et al.*, 1992) can be applied to microsatellite data (Michalakis and Excoffier, 1996) to obtain ϕ_{ST}, an analogue of Slatkin's (1995) R_{ST} statistic. Two representative haplotypes were defined for each sample, based on the composite genotype revealed by the four polymorphic loci (eg. composite genotype 1, characterised by AA, AB, CC, AB for the four polymorphic loci, would be represented by haplotypes AACA and ABCB in an AMOVA). A matrix of squared Euclidean distances was constructed for all pairwise comparisons of all haplotypes. In order to simulate the R_{ST} statistic, distances were calculated as the sum of squared differences in allele size over all loci, such that $d_{xy}=\Sigma(a_{ij} - a_{i'j})^2$, where a_{ij} is the size of the ith allele at the jth locus for haplotypes x and y. Frequencies were determined by counting the number of individuals displaying each haplotype in each designated population.

Assuming that the microsatellite loci tested are statistically independent, random permutation of representative haplotypes would over-estimate the significance of measured ϕ statistics (Michalakis and Excoffier, 1996). Thus, the significance of both ϕ-statistic and F_{ST} values was determined using the relationship $\chi^2 = 2N_TF_{ST}$ with (s-1) degrees of freedom, where N_T is the total number of samples from s populations (Workman and Niswander, 1970).

5.2.4.2 Gene flow

5.2.4.2.1 Estimation of gene flow from mtDNA

Provided Euclidean distances are used, the ϕ_{ST} value generated by AMOVA is analogous to the G_{ST} of Takahata and Palumbi (1985) (Excoffier *et al.*, 1992). Thus $\phi_{ST} = 1/(1+2Nm_f)$ (after Takahata and Palumbi, 1985). Mitochondrial DNA gene flow was estimated from this equation as the number of migrant females occurring among populations and regions per generation, Nm_f.

5.2.4.2.2 Estimation of gene flow from microsatellite DNA

Gene flow (Nm) was calculated from the average F_{ST} over all loci using the relationship $F_{ST} = 1/(1+4Nm)$ (Wright, 1951; Hartl, 1988). The R_{ST} analogue, ϕ_{ST}, was used to estimate M_R from the equation $M_R = [(d_s - 1) / 4d_s] (1 / R_{ST} - 1)$, between / among d_s populations (Slatkin, 1995).

5.2.4.3 Cluster analysis

Trees were constructed by the distance Wagner method and by cluster analysis using the unweighted pairgroup method with arithmetic averages (UPGMA). Pairwise distances were calculated using the simple Manhattan distance (Prevosti distance, Wright, 1978) and Nei's (1978) unbiased genetic distance.

5.2.4.4 Composition of schools

During the study, four separate schools were sampled using the biopsy method described in section 2.2.2.1. Although comprehensive data pertaining to age-sex classes could not be obtained, the gender of each sample was determined using the molecular method described in section 2.8 and the gender composition of each of the schools was assessed.

5.3 RESULTS

5.3.1 Geographic distribution of mtDNA control region haplotypes

Of the 10 haplotypes defined for east coast inshore bottlenose dolphins, two haplotypes accounted for 89.2% of the sample. Frequency differences at the single transition (see section 3.3.1 and Table 3.1) defining haplotypes 2 and 4, characterised samples collected from KwaZulu-Natal and the East Cape (Fig 5.1). Haplotype 4 occurred in 58% of the KZN samples, while haplotype 2 occurred in 61% of the East Cape samples. Each of the remaining eight haplotypes was found in a single individual, six of which occurred in the KZN sample and two in the East Cape sample.

5.3.2 Geographic distribution of microsatellite DNA genotypes

Overall, genotypes scored for the four polymorphic microsatellite loci did not display regional differentiation, although individual loci showed frequency differences within and among the KZN and East Cape regions. Both groups within the KZN region displayed the 256 homozygote (locus 415/416) in more than 75 % of the samples, while the 256 homozygote and 244/256 heterozygote were represented approximately equally in the East Cape sample (Fig. 5.2a). All rare genotypes scored for locus 199/200, were found in the KZN sample, while East Cape animals displayed homozygote fixation for allele 192. Finally, the frequency of heterozygotes at locus 409/470 increased linearly from north to south.

Following the suggestion by Goodwin *et al.* (1996) that East Cape bottlenose dolphins may move into the KZN region annually during the winter 'sardine run', the KZN north and south coasts were separated into samples taken from, inclusively, May to October (winter) and November to April (summer) and tested for seasonal differentiation. Genotype frequencies at three of four loci indicated similarity between the East Cape sample and dolphins taken during winter months off the KZN south coast. Genotype frequencies at locus 464/465 were approximately equal for the KZN south coast winter sample and the East Cape sample. Both the south coast and north coast winter samples showed a predominance of 138/140 heterozygotes as was seen in East Cape animals, while the summer samples displayed far fewer heterozygotes (Fig. 5.3b). Several genotypes occurred in low frequency for locus 199/200. However, the majority of these were restricted to the KZN north coast and south coast summer sample. The south coast winter sample displayed a preponderance of 192/192 homozygotes and only one other genotype, while the East Cape sample was fixed for the 192/192 homozygote (Fig. 5.3c). Finally, the winter samples from both the KZN south coast and the East Cape displayed similar frequencies for the 186/190 heterozygote at locus 409/470. Furthermore, the occurrence of the 190/190 homozygote was greater in the KZN north coast animals and the south coast summer sample, than in those taken from the south coast winter sample and East Cape animals (Fig. 5.3d).

Wagner and UPGMA trees based on both the Prevosti (Wright, 1978) and Nei's (1978) genetic distances revealed a similar relationship among seasonal divisions on the KZN coast (Fig. 5.4). A smaller genetic distance was found between the KZN south coast winter catch and the East Cape sample than with either the KZN south coast summer sample or the rest of the KZN coast. This relationship remained unchanged with the seasonal division of the KZN north coast sample.

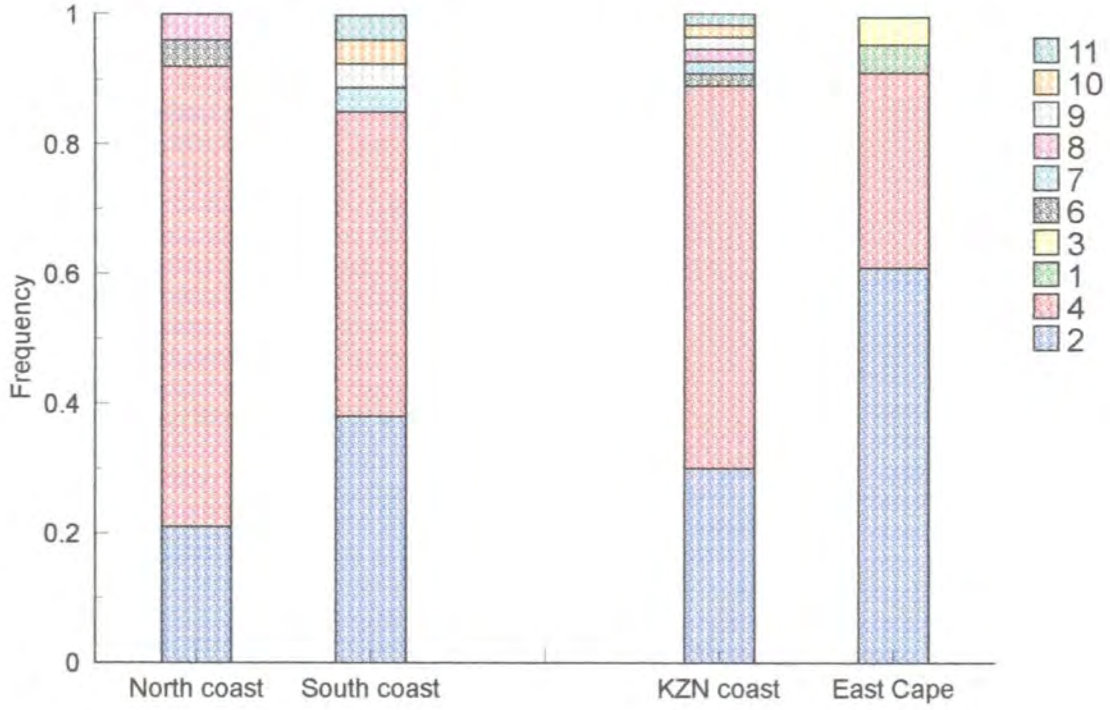


Figure 5.1 Geographic distribution of mtDNA haplotypes in bottlenose dolphins. The whole KZN coast is represented on the right, while separate data for the north and south coasts are presented on the left. Haplotype numbers correspond with those in Table 3.1

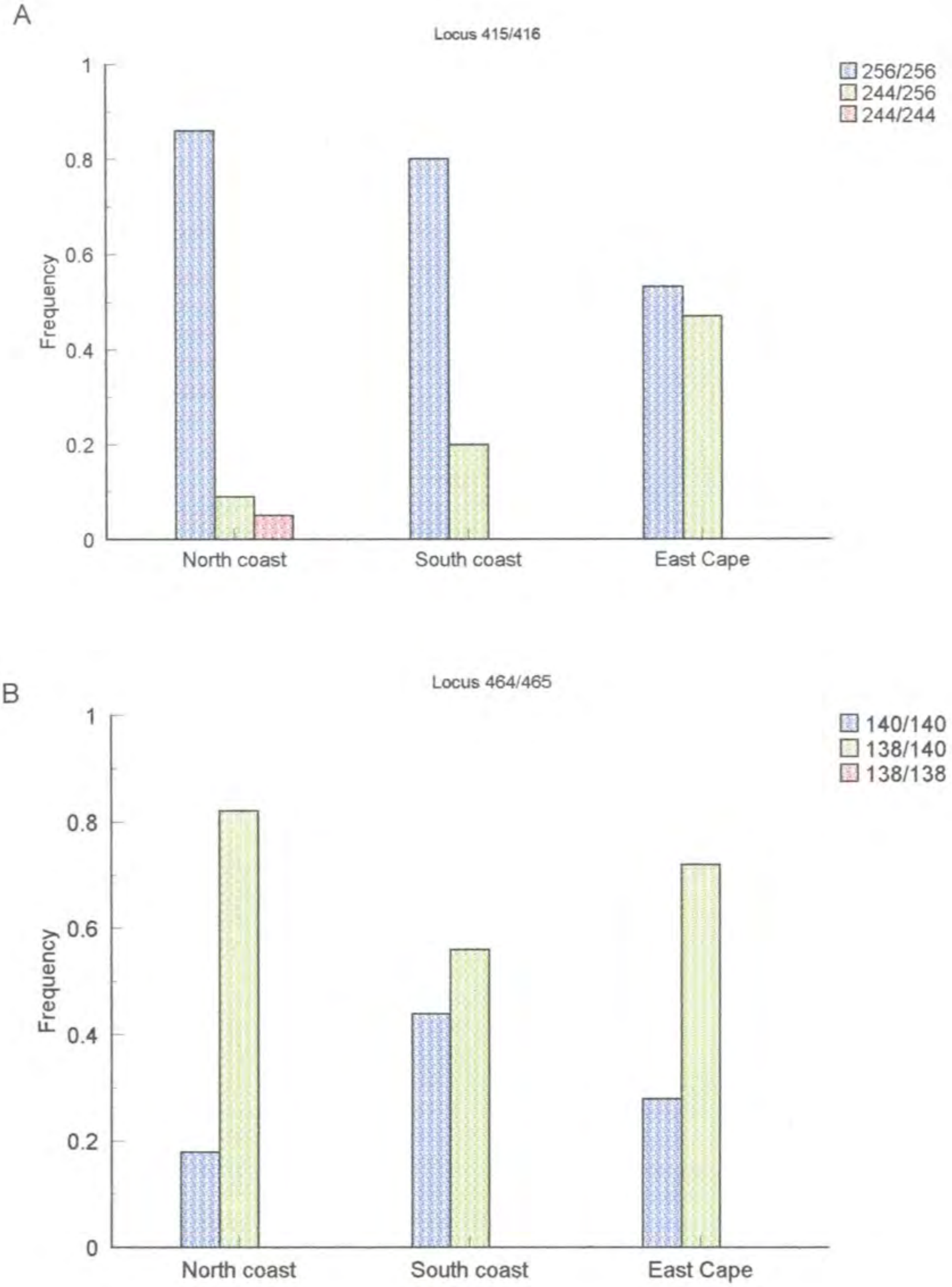


Figure 5.2 Geographic distribution of genotypes for four polymorphic microsatellite loci. Legends indicate genotypes according to allele size in base pairs.

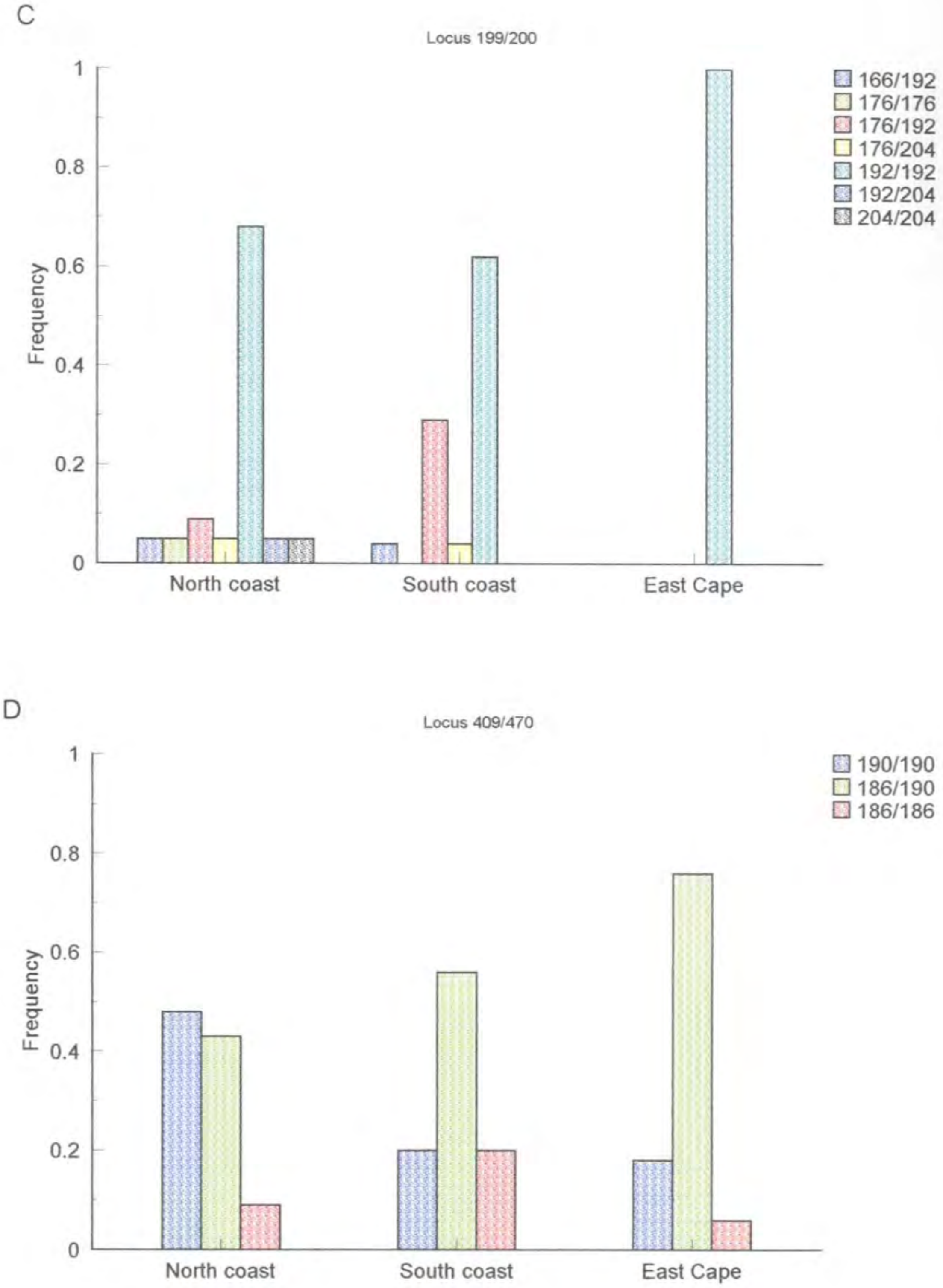
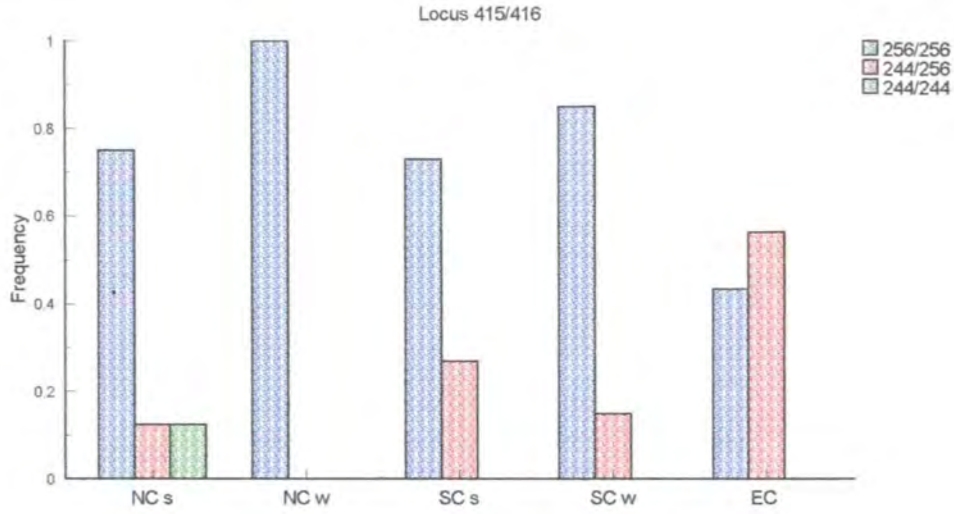


Figure 5.2 continued..

A



B

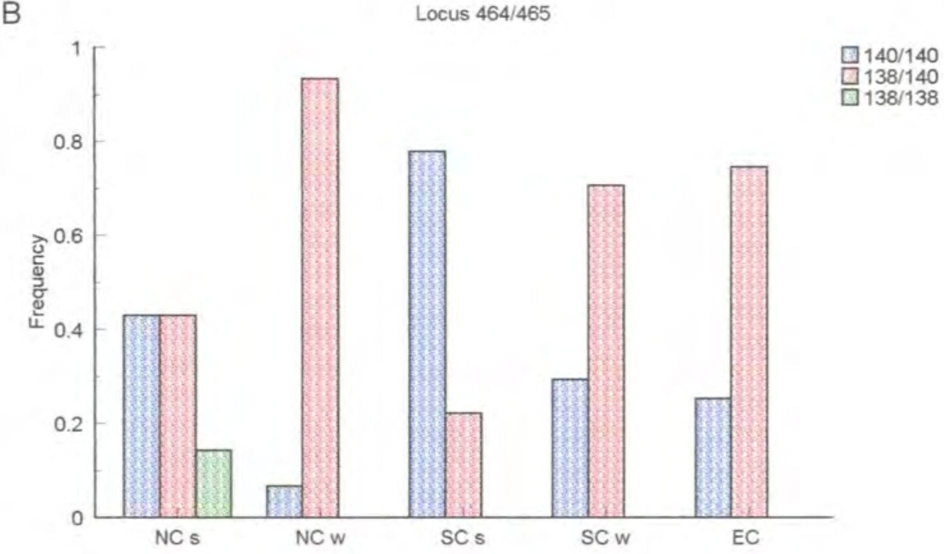
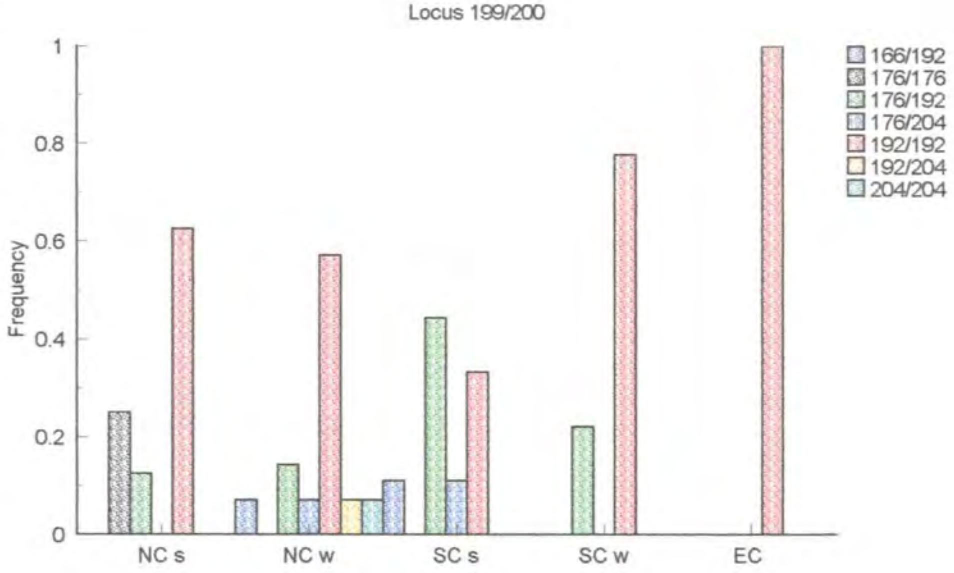


Figure 5.3 Seasonal distribution of KZN microsatellite genotypes. Lower case *s* and *w* represent samples taken during, respectively, summer and winter months, as described in the text.

C



D

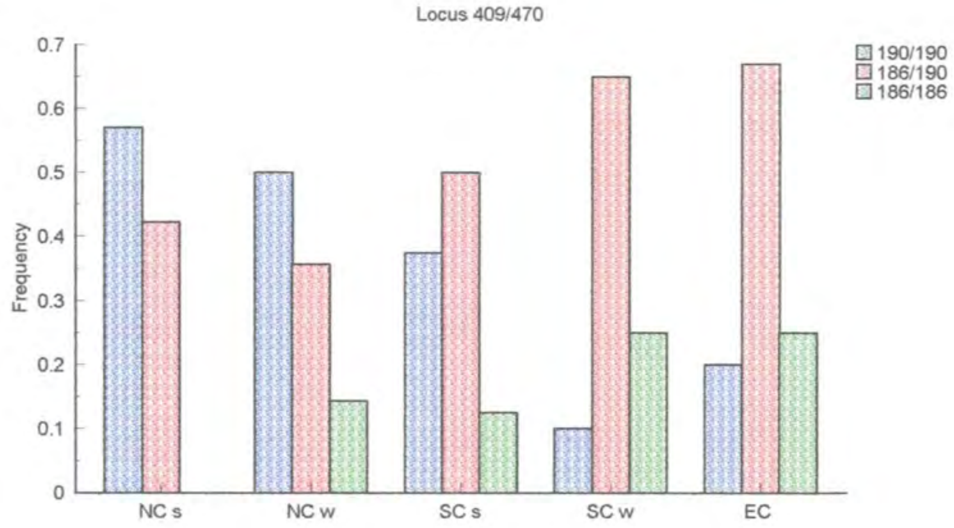


Figure 5.3 continued..

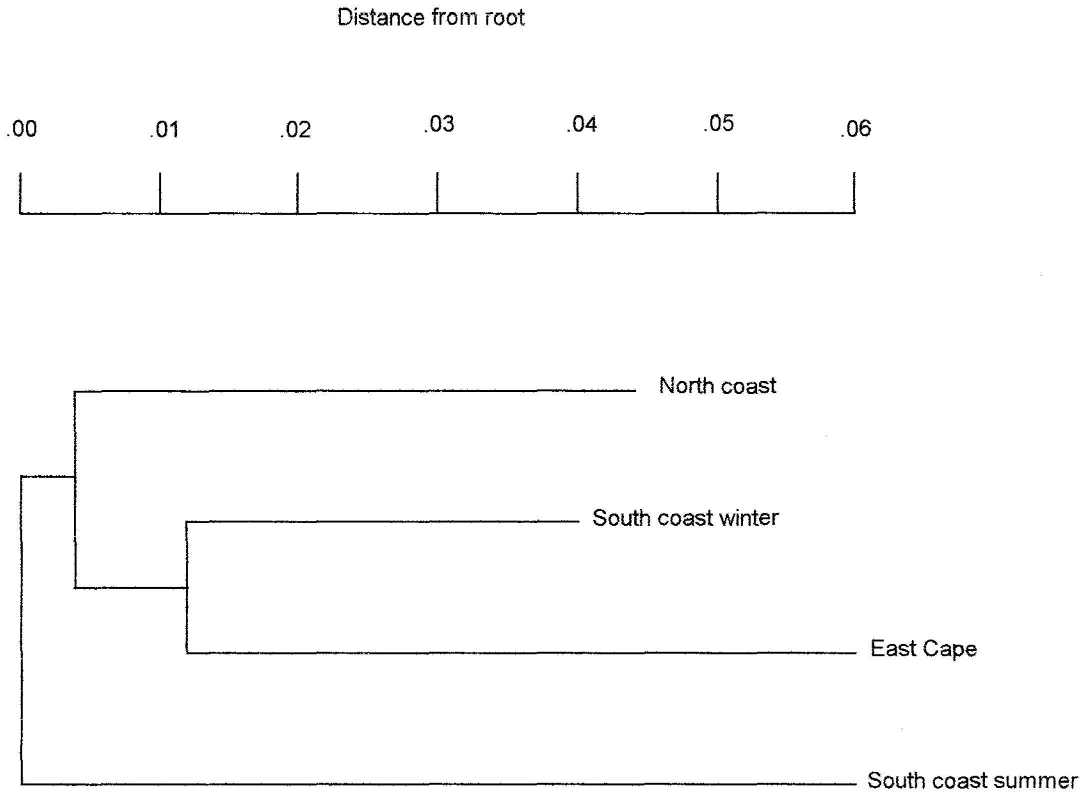


Figure 5.4 Wagner tree indicating the relationship between the temporally-divided KZN south coast and the remainder of the coast, based on allele frequencies.

5.3.3 Genetic differentiation estimated from mtDNA

AMOVA was first used to assess the degree of differentiation between the two major regions, KwaZulu-Natal and the East Cape. Molecular variance between the two regions accounted for 13.7 % of the total variance observed in the population, implying a significant degree of subdivision between the regions (Table 5.1). This differentiation reflects the distribution of the two major haplotypes between the KZN and East Cape populations as well as the relative number of rare haplotypes found in either region. Analysis of the three subgroups revealed no significant subdivision between the KZN north and south coasts, however, the division between KZN and the East Cape was supported. Furthermore, the level of genetic differentiation observed within the KZN region (ϕ_{SC}= 0.063; ϕ_{ST}= 0.075), implied a lack of significant subdivision among the six 'preferred areas' (Ross *et al.*, 1987; Cockcroft *et al.*, 1990b).

The whole sample was divided seasonally and by gender to determine whether the occurrence of the two major haplotypes could be subdivided on these bases. However, no molecular variance could be attributed to the sex or seasonal occurrence of individuals (ϕ_{SC} = -0.012 and -0.033, respectively; Table 5.1).

5.3.4 Genetic differentiation estimated from microsatellite DNA

The R_{ST} analogue, ϕ_{ST} was estimated following the recommendations by Michalakis and Excoffier (1996), and compared with the jack-knifed mean of F_{ST} values across all loci (Weir and Cockerham, 1984). Microsatellite DNA supported the differentiation of KZN and the East Cape, as indicated by mtDNA. Similarly, no significant differentiation was evident among the six 'preferred areas' within KZN (Table 5.2). In order to test microsatellite genotype variance for female philopatry, the KZN north coast and south coast samples were further divided by gender. However, almost no differentiation was noted between the sexes within either group (ϕ_{SC} = 0.004), suggesting homogeneity in nuclear DNA among males and females. Gender differentiation could not be determined for the East Cape sample, as a single female was sampled among the three schools (see section 5.3.7).

The seasonal grouping suggested by Figure 5.4 was tested for significance, using the AMOVA and F_{ST}. A ϕ_{SC} of 0.015 attributed only 1.5 % of the total differentiation to differences between the summer and winter catches within the KZN south coast sample. Moreover, the F_{ST} of 0.029 was not significant, implying that the level of differentiation suggested by that particular grouping was insufficient to indicate significant subdivision.

5.3.5 Gene flow and migration estimated from mtDNA

Despite indications by the AMOVA that significant mtDNA differentiation exists between the KZN and East Cape regions, gene flow estimates, calculated using the relationship of Takahata and Palumbi (1985; section 5.2.4.2.1) indicate that three females are exchanged per generation (Table 5.3). This exceeds the one migrant per generation required to prevent differentiation due to drift (Slatkin, 1987). The degree of differentiation, correlated with distance between regions (Table 5.3), indicated an increase in the migration rate with a decrease in geographic distance. The extent of gene flow, measured in terms of the number of female migrants, was highest between field and aerial survey-designated 'preferred areas' within KZN and lowest between the KZN and East Cape regional divisions. These data are indicative of an isolation by distance model of gene flow (Slatkin, 1993) and suggest a lack of strict local philopatry within the KZN region, with greater regional fidelity between KZN and the East Cape.

5.3.6 Gene flow estimated from microsatellite DNA

Similar estimates of gene flow were noted for R_{ST} and F_{ST}. Given the significant allele frequencies between the two major regions, a migration rate of 6.8 and 6.5 migrants per generation for R_{ST} and F_{ST}, respectively, were estimated. As was the indication from mtDNA, the migration rate among localities within KZN was estimated to be more than twice that between KZN and the East Cape (Table 5.3).

5.3.7 Gender determination of biopsy samples

Of the 29 individuals sampled using the biopsy method, 21 were determined to be male and eight were female. Low numbers and few sampling events prevent efficient statistical testing, however, a few points regarding the composition of schools are noteworthy. A single school was sampled off the KZN coast during the winter months. During a single sampling event, skin biopsies were obtained from 11 individuals, seven of which were female and four of which were male. Of a total of 18 individuals sampled from three schools off the former Transkei coast and Algoa Bay, 17 were male, while only a single female was sampled.

A single supposed mother-calf pair was analysed. The relationship was presumed from the simultaneous occurrence of a lactating female and a calf (Cockcroft and Ross, 1989) in a shark net. Analysis of the mtDNA from both samples revealed identical maternal haplotypes. Although this increases the probability of a familial relationship between the two animals, it does not confirm that they were, in fact, mother and calf (cf section 7.3.5).

Table 5.1 Results of an AMOVA of mtDNA differentiation in South African bottlenose dolphins. KZN = KwaZulu-Natal, EC = East Cape, NC = KZN north coast, SC = KZN south coast. Variance components correspond with those given in Excoffier *et al.* (1992)

Groups	Populations	Variance component	% total variance	ϕ statistic	Permutations	p value
Whole population	KZN	AP	13.72	$_{ST}$ 0.137	2 000	0.0035
	E C	WP	86.28			
KZN	North coast	AG	9.67	$_{CT}$ 0.097	2 000	< 0.0050
	South coast	AP/WG	3.43	$_{SC}$ 0.038		0.1049
E C	East Cape	WP	86.90	$_{ST}$ 0.131		< 0.0050
NC	Preferred areas 1-3	AG	1.23	$_{CT}$ 0.012	1 000	0.3178
		AP/WG	6.27	$_{SC}$ 0.063		0.1124
SC	Preferred areas 4-6	WP	92.50	$_{ST}$ 0.075		0.0820
KZN	Male	AG	10.06	$_{CT}$ 0.101	2 000	< 0.0010
E C	Female	AP/WG	-1.11	$_{SC}$ -0.012		0.5045
		WP	91.06	$_{ST}$ 0.089		< 0.0010
KZN	Summer	AG	8.83	$_{CT}$ 0.088	2 000	0.1958
E C	Winter	AP/WG	-2.97	$_{SC}$ -0.033		0.8841
		WP	94.15	$_{ST}$ 0.059		0.1958

Table 5.2 Results of an AMOVA of genetic differentiation in microsatellite DNA in South African bottlenose dolphins. The ϕ statistic is analogous to Slatkin's R_{ST} (1995). Variance components estimated by AMOVA are as described in Table 5.1. Significance testing of both the R_{ST} analogue, ϕ_{ST}, and F_{ST} was performed following Workman and Niswander (1970) and is presented as χ^2 values. NC = north coast, SC = south coast. A single asterisk denotes significance to $p<0.05$ and a double asterisk denotes significance to $p<0.025$.

Groups	Populations	Variance component	% total variance	ϕ statistic	χ^2 value (df)	F_{ST}	χ^2 value (df)
Whole population	KZN	AP	1.76	$_{ST}$ 0.018	5.184 **	0.037	3.996 *
	E C	WP	98.24		(1)		(1)
KZN	North coast	AP	1.10	$_{ST}$ 0.001	0.216	0.031	3.348
	South coast	WP	98.90		(1)		(1)
KZN	preferred areas 1-3	AG	-0.63	$_{CT}$ -0.006	1.296	0.017	2.448
	preferred areas 4-6	AP/WG	1.96	$_{SC}$ 0.020	4.320		(5)
		WP	98.67	$_{ST}$ 0.013	2.808		
					(5)		
N C	Male	AG	-0.14	$_{CT}$ -0.001	0.216	0.001	0.108
S C	Female	AP/WG	0.37	$_{SC}$ 0.004	0.864		(1)
		WP	99.76	$_{ST}$ 0.002	0.432		
					(3)		
KZN North coast	Summer	AG	1.81	$_{CT}$ 0.018	5.184	0.024	3.456
KZN south coast	Winter	AP/WG	-0.34	$_{SC}$ -0.003	0.864		(5)
E C		WP	98.53	$_{ST}$ 0.015	4.320		
					(5)		
KZN North coast	N. coast	AG	-0.70	$_{CT}$ -0.007	2.016	0.029	4.176
KZN south coast	S. coast (summer)	AP/WG	1.50	$_{SC}$ 0.015	4.320		(3)
	S. coast (winter)	WP	99.20	$_{ST}$ 0.008	2.304		
E C	E C				(3)		

Table 5.3 Population subdivision and gene flow, estimated as the number of migrants per generation from mtDNA haplotype data (ϕ_{ST}) and microsatellite allele frequency data (F_{ST} and R_{ST}). Probability values of ϕ_{ST} and F_{ST} are given in Table 5.2.

Populations	Population subdivision and gene flow estimates						
	Mitochondrial DNA		Nuclear microsatellite DNA				
	ϕ_{ST}	Nm_f	F_{ST}	Nm	R_{ST}	M_R	d_s
KZN - East Cape	0.137	3.15	0.037	6.507	0.018	6.819	2
KZN north coast - KZN south coast	0.075	6.17	0.031	7.815	0.001	124.88	2
KZN preferred areas	$0.063^{‡}$	7.44	0.017	14.46	0.013	15.79	6

‡ ϕ_{SC} = variance among populations within the two groups

5.4 DISCUSSION

5.4.1 Phylogeographic population structure

Mitochondrial DNA phylogenetic data can be used qualitatively to describe overall patterns of population structure. Avise *et al.* (1987) specified five categories describing intraspecific phylogenies overlaid on geographical distribution. Of these, category V species or populations are characterised by a broad geographic distribution of some haplotypes, while related haplotypes are localised, displaying a parapatric distribution. Additional properties include the widespread occurrence of presumed ancestral haplotypes and localisation of new mutations. The phylogeographic continuity of the two major haplotypes as well as the localisation of related unique haplotypes and partial spatial separation of KZN and East Cape bottlenose dolphins suggests that the category V classification (Avise *et al.*, 1987) best describes this population.

The criteria used by Avise *et al.* were modified by Dizon *et al.* (1992) to include differences in the locally adapted genome rather than merely mtDNA distance. Their classification accounted for differences in demographic, morphological, nuclear or mtDNA parameters which may indicate differential selection in natural populations. Under these criteria, bottlenose dolphins in this study comply with the characteristics of category IV populations. These include a high level of gene exchange in the absence of geographical barriers, as suggested by greater estimates of gene flow from microsatellite data. Under these criteria, populations may show no evidence of differentiation and may, therefore, be effectively panmictic. However, in risk-averse management, such as for the purpose of conservation, they may still be regarded as separate stocks (Dizon *et al.*, 1992).

5.4.2 Genetic structure and composition of schools

All but one of the samples taken from bottlenose dolphin schools in Algoa Bay and Transkei were male. Results from nuclear, mitochondrial and random primed DNA confirm that no two samples originated fom the same animal, suggesting either the occurrence of exclusively male schools (section 3.4.2.2) or merely that in mixed schools, males are more likely to be sampled than females.

A lack of sexual dimorphism in bottlenose dolphins hinders the identification of gender in the field. Consequently, no observational data are available which describe the gender composition of bottlenose dolphin schools on the South African coast. However, incidentally-captured bottlenose dolphins in KZN can be segregated into different age-sex groups on the basis of prey taken (Cockcroft, 1990b, 1992; Cockcroft and Ross, 1991). The distribution of these prey species within the inshore zone implies that different age-sex groups of bottlenose dolphins occur separately in different inshore areas. For example, the stomachs of lactating females and calves have been found to contain inshore benthic prey (Cockcroft and Ross, 1990) and they are the only classes which feed on pilchards (op cit.), demonstrating a strictly inshore distribution. By contrast, mature males are the only age-sex class to feed on *Priacanthus* species which inhabit deeper coastal waters (op cit.).

Discrete male and female groups have been well-documented in parts of the bottlenose dolphin's range (Wells *et al.*, 1980; Scott *et al.*, 1990; Würsig and Harris, 1990). Evidence that each age-sex class of bottlenose dolphin prefers different prey sizes and in some cases, different species, suggests that large schools may disassociate into subgroups containing animals of a certain age-sex class which utilise different regions of the inshore zone. If these schools remain fragmented for extended periods, it is likely that sampling a particular subgroup will bias the sample towards that age-sex class. This demonstrates the need to further investigate the composition of South African coastal bottlenose dolphin schools. Knowledge of the stratification of age-sex classes within the inshore zone may assist in determining the longshore mobility or philopatry of the species and whether either is strictly characteristic of certain age-sex classes.

5.4.3 Local genetic differentiation and philopatry within KwaZulu-Natal

In parts of its range the inshore bottlenose dolphin displays fidelity to a restricted area which comprises its home range (Saayman *et al.*, 1973; Würsig and Würsig, 1979; Shane, 1986; Scott *et al.*, 1990; Weigle, 1990; Würsig and Harris, 1990). Peaks in sighting rates (Ross *et al.*, 1987; Cockcroft *et al.*, 1990b) led to the proposal that dolphins on the KZN coast occupied 'preferred areas' which represent home ranges spanning some 30 - 40 km in length (Cockcroft *et al.*, 1990b). Repeated sightings of several individuals within the same areas in KZN throughout a three year study period suggests that at least some dolphins preferentially inhabit particular areas (Peddemors, 1995). However, mtDNA and microsatellite data suggest that strict fidelity to the proposed 'preferred areas' is not observed by bottlenose dolphins. This may not apply to all dolphins in an area. Rather, certain groups of animals may be less mobile than others. Females and young calves, for example, may reside in 'nursery areas' (Ross and Cockcroft, 1990) within a defined longshore region (Irvine *et al.*, 1981; Würsig and Harris, 1990). The extent of this range would be restricted both by food availability and by the strength of calves and would, presumably, not extend beyond the 30 - 40 km spanning a 'preferred area'. With a larger sample, it may be possible to analyse females and calves separately, to test for local site fidelity. However, in this study, the inclusion of mature males, which are assumed to represent the vectors of gene exchange, would undoubtedly have obscured any significant structure suggested by the less mobile age-sex classes.

In light of the lack of subdivision in KZN, an important caveat should be noted. The collection of samples from incidentally-captured dolphins presents empirical difficulties when testing the genetic discreteness among dolphins from different 'preferred areas'. Analysis revealed that peaks in bottlenose dolphin capture are offset by approximately 35 km from the sighting peaks used to propose the six 'preferred areas' (Cockcroft, 1990b; Cockcroft *et al.*, 1990b). This distance approximates the length of a single 'preferred area' and led to the suggestion that dolphins are not captured within their 'preferred area', rather, in adjacent localities with which they are unfamiliar (Cockcroft, 1990b; Cockcroft *et al.*, 1990b). This would imply that dolphins caught in shark nets would not necessarily be representative of schools resident in the vicinity in which they are caught and would, therefore, be unsuitable samples for testing the genetic relationships among suspected 'preferred areas'. It is possible, therefore, that the lack of significant structure seen among local groupings of bottlenose dolphins within the KZN region is an artifact of including in the sample, animals from adjacent localities. However, without further genetic data from known residents in a particular 'preferred area', this remains mere speculation.

These difficulties may be overcome using a combination of genetic analysis and photo-identification mark-recapture analysis of whole schools to best describe the level of residency within and discreteness among putative 'preferred areas'. Currently, sample collection and photo-identification mark and recapture are being undertaken by the Natal Sharks Board (V.M. Peddemors, personal communication). It is hoped that data from those samples will clarify the question of local residency and site fidelity.

5.4.4 Regional genetic differentiation and philopatry

Mitochondrial DNA haplotype frequency characteristics support a division between the KZN and East Cape regions (Fig. 5.1) and in all groupings in an AMOVA, the among groups component (AG) is significant (Table 5.1), confirming that significant mtDNA genetic variance exists between the two regions. Three female migrants per generation between KZN and the East Cape is sufficient to obviate the effects of genetic drift and to ensure that the same haplotypes are shared between the two regions, however, it is probably not sufficient to maintain identical haplotype frequencies between them (Allendorf and Phelps, 1981). Thus, statistically significant divergence is evident even though there is mtDNA exchange between the regions. This structure suggests that female bottlenose dolphins tend towards regional philopatry. Significant R_{ST} and F_{ST} values (Table 5.2) supported the nuclear differentiation implied by variations in gene frequency between KZN and East Cape dolphins (Fig. 5.2). Furthermore, gene flow estimated between the two regions from both nuclear and mtDNA was approximately half that determined for localities within KZN (Table 5.3). This implies that although homogeneity is indicated within KZN, genetic exchange between it and the East Cape is sufficiently limited to suggest that both male and female bottlenose dolphins show some fidelity to either region.

Coastal marine mammal populations display varying degrees of genetic differentiation over their range. Regional mtDNA differentiation was also found in bottlenose dolphins along the Atlantic coast of the United States (Dowling and Brown, 1993) and populations of harbour seal (*Phoca vitulina*), while showing local movement within regions, displayed different haplotype frequencies among regions along the Pacific coast (Lamont *et al.*, 1996; Stanley *et al.*, 1996). By contrast, bottlenose dolphins sampled in the Gulf of Mexico gave indications of considerable gene exchange among populations (Dowling and Brown, 1993). Similarly, populations of California sea lion (*Zalophus californianus*) displayed considerable maternal gene flow along the southern California coastline and striped dolphins (*Stenella coeruleoalba*) showed no evidence of differentiation along a region of Mediterranean coastline approximately equal in length to the coastline in this study (Garcia-Martinez *et al.*, 1995).

Whether or not bottlenose dolphins from the KZN coast and the Algoa Bay region of the East Cape should be considered separate populations depends on several factors relating to their genetics, demographics and management. However, it is likely that the coastal region under study comprises at least two stocks. Without thoroughly sampling localities along the entire coastline it is not possible to speculate on the extent of home ranges or to accurately subdivide the entire South African population. However, it does appear that dolphins sampled in the gillnet-stressed KZN region are, at least maternally differentiated from those in the adjacent, unstressed East Cape.

5.4.5 Seasonal exchange and the 'sardine run' migration

Despite significant genetic heterogeneity between KZN and the East Cape, there is still sufficient gene flow to prevent isolation of the regions by genetic drift. However, it would appear that a regular migration event would be necessary to facilitate consistent gene flow over distances of 500 km or more. This may occur during the annual 'sardine run' (Cockcroft, 1990b; Peddemors, 1995), a seasonal migration of the pilchard *Sardinops sagax* from the southern and western Cape towards Durban (Fig. 5.5).

The migration is facilitated by the expansion of pilchard habitat during winter with the sporadic insurgence of cold water into the warmer Indian Ocean coastal zone

(Heydorn *et al.*, 1978; Armstrong *et al.*, 1991). Current reversals result from wind forcing, while wave action in the inshore zone generates a longshore drift which the sardines use to move north eastwards (Schumann, 1987). During this time, the sardines are followed by an influx of piscivorous birds and predatory teleosts (Heydorn *et al.*, 1978). Many of the teleost species taken by bottlenose dolphins are also associated with the migration. Of these, the haemulid, *Pomadasys olivaceum* is by far, the most important prey item taken by South African bottlenose dolphins (Cockcroft and Ross, 1990). Along with the pilchard, this species is important prey for the bluefish, *Pomatomus saltatrix* (Heydorn *et al.*, 1978), which, together with the sparid *Sarpa salpa* and the sciaenid *Argyrosomus hololepidotus* comprises a large proportion of the bottlenose dolphin winter prey spectrum (Cockcroft and Ross, 1990).

Every year, large numbers of bottlenose dolphins are seen following the migration between Algoa Bay and Durban (Peddemors, 1995). During a three-year study a minimum of 2000 bottlenose dolphins were present off the Transkei coast (Fig. 5.5) during peak abundance and were reported to represent a migratory East Cape stock (Peddemors, 1995). Bottlenose dolphins in other regions have been known to shift their distribution in response to prey abundance (Weigle, 1990; Scott *et al.*, 1990). Moreover, coastal bottlenose dolphins are capable of covering the distance required to follow the 'sardine run'. Würsig and Würsig (1977) reported a 600 km round trip completed by coastal bottlenose dolphins in the south Atlantic and Wells *et al.* (1983) documented a 1500 km round trip for identifiable dolphins off southern California.

An enigmatic aspect of the bottlenose dolphins' association with the 'sardine run' is their disappearance from the coastal region at Ifafa, on the KZN south coast (Peddemors, 1995) (Fig. 5.5). Baird (1971) has suggested that once the pilchard shoals reach their most northern distribution around Durban, they move offshore into the southward-moving Agulhas Current and return to the southern and western Cape. Goodwin *et al.* (1996) have suggested that bottlenose dolphins may also use the fast-flowing Current to return to the East Cape. The Agulhas Current, which normally lies close inshore, moves further offshore between Port Durnford (north coast) and Green Point (north of Ifafa) (Fig. 5.5) in response to the widening continental shelf (Heydorn *et al.* 1978). Over this range, it meanders for great distances and is characterised by cyclonic eddies which form inshore of the current boundary (op cit.). If bottlenose dolphins make use of the current's southward movement, it is likely they would do so while it can be found close inshore and before it became associated with other disturbances.

Microsatellite allele frequency data support the hypothesis of East Cape dolphins following the pilchard migration only as far as the KZN south coast. Dolphins caught on the KZN south coast during the winter months of the migration display similar allele frequencies to and cluster more closely with those sampled in the East Cape (Figs. 5.3 and 5.4). This would suggest that dolphins from both the south coast and East Cape are being captured in shark nets on the KZN south coast during the migration. This result may assist in interpreting the subdivision of the KZN north and south coasts implied by allozyme allele frequencies (Goodwin *et al.*, 1996) and organochlorine loads (Cockcroft *et al.*, 1989b). It is likely that, in both studies, the south coast sample comprised dolphins captured during the 'sardine run', some of which may have been East Cape 'migrants'. Differences in allele frequency and organochlorine load between KZN south coast and East Cape dolphins would have been ignored in analysis. However, those differences may have been sufficiently large to differentiate the combined south coast-East Cape sample from the KZN north coast sample. Without a way of discerning between dolphins which may be resident on the south coast and those which are migrants from the East Cape, it is not possible to determine how much of the estimated gene flow is a result of true genetic mixing and how much is an artifact of capturing and sampling two different stocks on the south coast. Thus, sampling net-captured dolphins may under-estimate the discreteness of proposed KZN and East Cape stocks.

The inclusion of a neonate offshore bottlenose dolphin in the sample collected from KZN shark nets (see section 4.3.1) would imply the presence of adults of the offshore form in the nearshore zone. The entanglement of the calf during the month of June, at the height of 'sardine run' activity, poses the question of whether offshore bottlenose dolphins are also attracted to the abundant food resources associated with the migration. Common dolphins (*Delphinus delphis*) which are found offshore for the remainder of the year, are abundant in nearshore regions during the 'sardine run' (Cockcroft, 1990b; Cockcroft and Peddemors, 1990; Cockcroft *et al.*, 1990a), where they feed primarily on pilchards (Cockcroft and Ross, 1983). If offshore bottlenose dolphins do move inshore during the 'sardine run', it may present a great potential for gene exchange with inshore dolphins. Although Ross (1977) found no indication of hybridisation between inshore and offshore bottlenose dolphins from morphological data, successful mating has occurred between offshore forms from the South African west coast and inshore forms from the east coast in captivity at Sea World, Durban, indicating the possibility for hybridisation in natural populations.

The biota involved with the annual 'sardine run' are major role players in the ecology of the east coast. It is possible that the migratory behaviour of predatory fish evolved in response to the annual increased abundance of pilchards (Armstrong *et al.*, 1991). This may well be the case with bottlenose dolphins on the east coast. A decline in the intensity of the 'sardine run' has been reported as far back as 1970 (Baird, 1970). This was undoubtedly a result of the collapse of substocks of pilchard off South Africa in the early 1960s and off Namibia in the early 1970s (Shelton, 1992) as well as increased competition from the anchovy

(Baird, 1970). Nonetheless, pilchard stocks continue to be intensely exploited in the south east Atlantic. This bodes ill from a fisheries perspective, as conservation of these and other planktivores as forage species for more valuable hake stocks has not been given high management priority (Shelton, 1992). The effects of this may eventually extend to the ecology of the east coast. If the annual pilchard migration provides the only opportunity for gene flow between the East Cape and KZN bottlenose dolphins, then collapse of the pilchard population and eventual cessation of the migration may, in time, lead to isolation of these regional stocks.

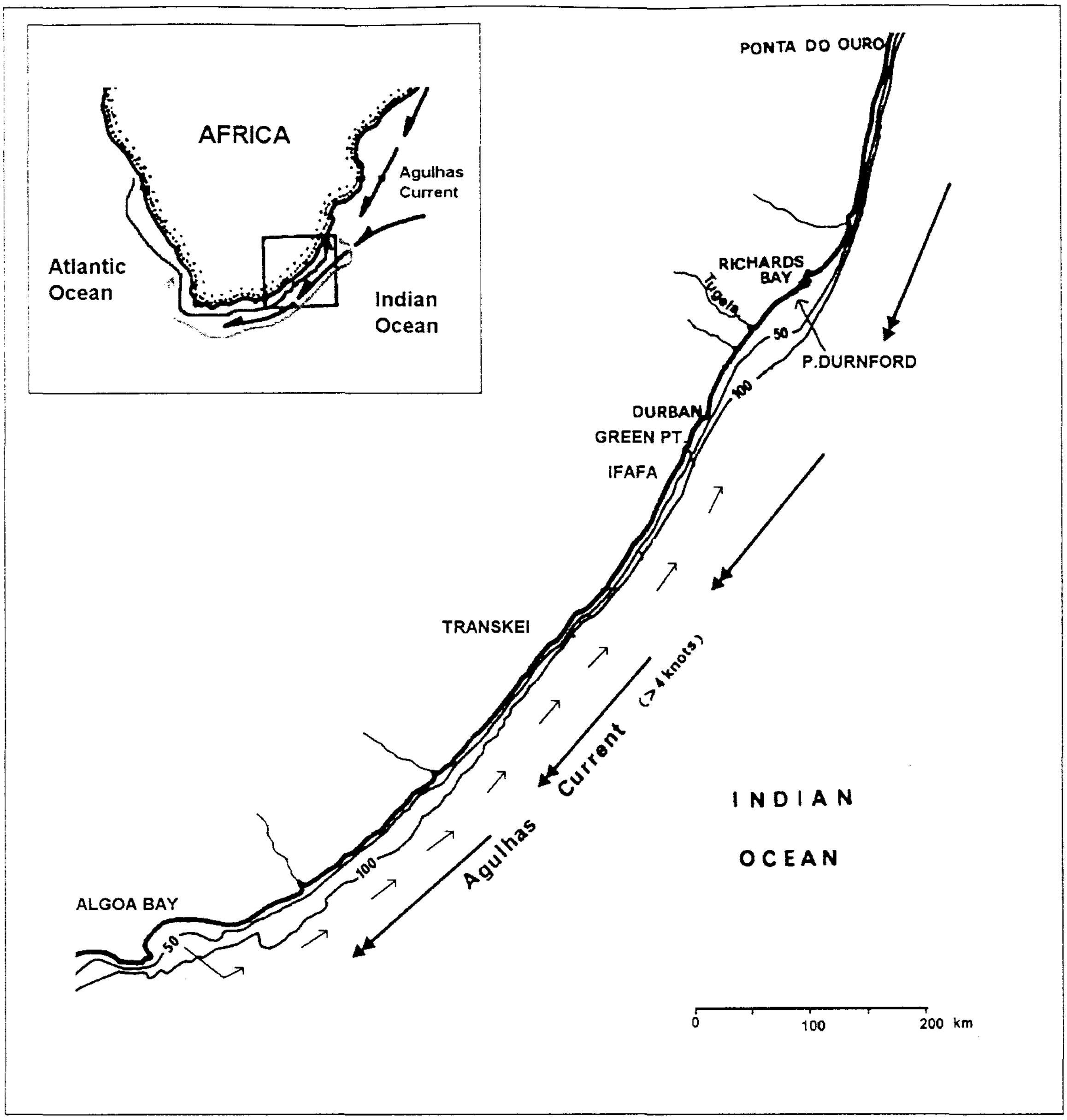


Figure 5.5 Annual 'sardine run' route and migration of bottlenose dolphins from Algoa Bay to the KZN south coast. The inset illustrates the migration route of the pilchard shoals from the south west coast to Durban. The first half of the return route follows the direction of the Agulhas Current. Small arrows extending from Algoa Bay to Ifafa indicate the proposed route followed by bottlenose dolphins in pursuit of the pilchard shoals. The 100m isobath indicates the edge of the continental shelf (modified from Heydorn *et al.*, 1978).

5.5 CONCLUSIONS

Peddemors (1995) noted that the "inclusion of non-resident dolphins in the [winter] catch presents a better picture for the sustainability of resident bottlenose dolphins in [KwaZulu]-Natal". However, the effects on sustainability lie not in the number of individuals caught, but in the contribution of those individuals to the persistence of the population.

By virtue of the inshore distribution of their preferred prey types, female bottlenose dolphins and the calves associated with them stand the greatest risk of capture in shark nets. This has been confirmed, where, over an 8-year period, 68% of the 212 bottlenose dolphins caught and analysed were mature females and young calves (Cockcroft and Ross, 1991). Of the total number of females caught, 75% were lactating or pregnant (op cit.). If females are philopatric, as estimates of mtDNA differentiation and gene flow suggest, it is unlikely that sufficient recruitment of females into KZN would occur from the adjacent East Cape region. Thus, despite the possibility that the annual 'sardine run' may facilitate gene flow between the two regions, this annual influx may not offset the mortality occurring in the netted region of KZN. Without recruitment, sustained shark net capture of two demographically important age-sex classes in KZN will gradually deplete the KZN stock.

However, although KZN and East Cape bottlenose dolphins do not appear to comprise a single stock, the former may be continuous with bottlenose dolphins to the north of the netted region. If the whole of the KZN coast comprises a single population, then it is possible that recruitment would occur from those areas to the north. This may be unlikely as bottlenose dolphins appear to avoid the turbid water which often prevails along the Tugela Bank (Ross, 1977; Cockcroft, 1992; see Fig. 7.4). Furthermore, a lack of bottlenose dolphin captures in the Richards

Bay shark nets (Cockcroft, unpublished data; Fig. 7.4), suggests that regular movement of bottlenose dolphins along the coastal Tugela Bank region is unlikely.

Future investigation would require additional samples of 'resident' KZN dolphins as well as additional samples from East Cape schools to confirm the subdivision proposed in this study. Furthermore, investigation of schools north of the Tugela Bank is urgently required to determine the extent of the proposed KZN stock. However, regular recruitment from the East Cape into KZN should not be relied upon to offset incidental captures in the latter.

PART II

CHAPTER 6

GENETIC DIVERSITY AND MITOCHONDRIAL DNA PHYLOGENY OF S.E. AFRICAN HUMPBACK DOLPHINS

6.1 INTRODUCTION

The threat of reduction of the species to relict populations has initiated priority research into the population dynamics of the humpback dolphin (Durham, 1995; Karczmarski, 1996; Obura *et al.*, 1996). This study marks the first molecular genetics investigation of the species or any of its populations. The east coast of South Africa represents the south western extreme of the range of the Indopacific humpback dolphin, is possibly the most intensively studied region of the species' distribution (Saayman and Tayler, 1979; Cooke, 1991; Durham, 1995; Karczmarski, 1996) and is probably the only area along the African east coast where the species is not threatened by extensive habitat destruction and deliberate harvesting (Cooke, 1991; Obura *et al.*, 1996).

Nonetheless, the persistence of the humpback dolphin off the coast of KZN is also threatened. Incidental capture in shark nets accounts for an annual mortality of up to 4% (Cockcroft, 1990b) of the estimated 160-200 animals (Ross, 1982; Durham, 1995) off the KZN coast. Exploitation in shark nets has occurred for more than 40 years (Cockcroft, 1990b; Cockcroft *et al.*, 1991b), the effects of which are becoming the focus of concern. This depletion is compounded by the susceptibility of humpback dolphins to shark attack (Cockcroft, 1991) and the accumulation of large amounts of organochlorine pollutants, a factor which may inhibit reproductive efficiency (Subramanian *et al.*, 1987; Cockcroft *et al.*, 1991a).

Recently researchers have emphasised the need for effective management and conservation of the species in South African waters (Cockcroft, 1990a; Durham, 1995; Karczmarski, 1996). Knowledge of the genetic structure and dynamics of the species is cursory, with photographic mark-recapture and field observations representing the principal methods of data collection along the South African coast (Saayman and Tayler, 1979; Durham, 1995; Karczmarski, 1996). These studies provide valuable information on the range and social dynamics of the species within a limited study area. However, they do not allow for investigation of long term genetic interaction, nor do they allow inferences to be made with regards to genetic subdivision and gene flow.

6.1.1 Genetic variability in South African humpback dolphins

As a small, exploited population at the edge of the species' range, South African humpback dolphins face depletion and possibly, local extinction if the population becomes fragmented and isolated. A preliminary genetic study indicated low allozyme variation in incidentally caught KZN humpback dolphins (Smith, 1990), but a small sample, combined with low molecular resolution of the technique suggested that analysis of the DNA would be necessary to uncover additional variability.

6.1.2 Objectives

Genetic variation in humpback dolphins from KZN and localities further south is assessed and discussed in light of available demographic and observational data. As this comprises the first comprehensive genetic study of any of the species' populations, additional samples were included from a geographically vicariant population, as a means of comparing levels of variability between populations within the species. The following measures of variability were assessed:

i) Nucleotide diversity in a portion of the mtDNA control region,
ii) Variation at six microsatellite loci ,
iii) Variation in random amplified polymorphic DNA (RAPD).

Applicable data were used in the estimation of:

i) Effective population size,

ii) Sequence divergence,

iii) Phylogeny of mtDNA haplotypes.

6.2 MATERIALS AND METHODS

6.2.1 Sample collection

Samples were collected from 30 humpback dolphins which were incidentally captured in shark nets along the KZN coast. A single sample was obtained from each of Algoa Bay, Maputo Bay and False Bay. Microsatellite analysis was not performed on the Maputo Bay sample, as the amount of DNA extracted from the specimen was sufficient only for mtDNA sequence analysis. Ten samples were obtained from humpback dolphins off the coast of Hong Kong. A detailed description of sample collection is described in section 2.2.1.2 and 2.2.2.2.

6.2.2 DNA analysis

DNA extraction, amplification, sequencing and electrophoresis were conducted as described in sections 2.3 to 2.5. Microsatellite loci were amplified and resolved as outlined in section 2.6.

6.2.3 Terminology

'South Africa' includes only that region of the coastline lying within the borders of the country. 'Southern Africa' refers to both the South African and Maputo Bay coastline. For the purpose of this study, the KZN coast was divided into a northern and southern region with the division occurring at Durban. This subdivision has been maintained from previous studies which have divided the KZN coast on the basis of demographic differences (Cockcroft, 1990b, 1991). The northern region, termed the Tugela Bank, was further divided into the 'north coast' and 'Richards Bay' on the basis of the relatively large and possibly resident, group occurring at the latter (Durham, 1995). Single samples taken from the eastern and southern Cape were included with the two samples obtained from the KZN south coast.

6.2.4 Data analysis

6.2.4.1 Nucleotide diversity

Nucleotide diversity was determined as the average number of nucleotide substitutions per site in a population (Nei, 1987) and was calculated according to the relationship given in section 3.2.4.1.

6.2.4.2 Mitochondrial DNA sequence divergence

Sequence divergence was calculated as the number of net nucleotide substitutions between two populations (Nei, 1987) as described in section 4.2.3.1.

6.2.4.3 Phylogeny reconstruction

Phylogenetic analyses were based on three different methods. Parsimony analyses were performed using MEGA (Kumar *et al.*, 1993). Branch and bound searches were performed to identify all minimum-length trees.

A neighbour-joining tree was constructed (Phylip 3.5, Felsenstein, 1993) using the genetic distances determined with the Kimura 2-parameter model, with a gamma correction (sections 4.2.3.1 and 4.3.2.2). The statistical confidence of each node was determined by bootstrap analysis with 500 iterations (Felsenstein, 1985). In addition, MEGA (Kumar *et al.*, 1993) was used to construct a neighbour-joining tree using the simplified version of the minimum evolution method (Saitou and

Nei, 1987). The statistical confidence was tested using 1000 bootstrap resamplings (Felsenstein, 1985).

A maximum likelihood tree was constructed by the DNAML program in the PHYLIP (Phylogeny Inference Package) version 3.5 software package (Felsenstein, 1993), using empirical base frequencies. Global re-arrangement and the jumble option were used to improve the probability of finding the tree with the greatest likelihood.

6.2.4.4 Microsatellite variation

Genetic diversity, conformance to Hardy-Weinberg expectations and the coefficient of inbreeding were measured as described in section 3.2.4.2.

6.2.4.5 Effective population size

Long term effective population size was estimated according to the equation of Wilson et al. (1985), described in section 3.2.4.3. Short term effective population size was estimated according to the temporal method of Waples (1989, 1991), given in section 3.2.4.4.

6.2.4.6 RAPD analysis

Random amplified polymorphic DNA was analysed as described in section 2.7. Statistical analysis of RAPD was not possible due to the presence of inconsistent bands and the lack of variation in consistently-scored bands.

6.3 RESULTS

6.3.1 Mitochondrial D-loop sequence variation

The DNA sequence was resolved for the first 400 bp of the noncoding mitochondrial DNA control region, extending from the $tRNA^{PRO}$, but excluding it, to similarity block E in the conserved central region (Brown *et al.*, 1986; Southern *et al.*, 1988). Three variable sites defined four haplotypes in South African humpback dolphins and a fifth haplotype was resolved for the single Maputo Bay sample. All of the changes in haplotypes 1 to 4 were A<->G transition substitutions. Of the 11 sites variable between the South African consensus sequence and the Maputo Bay sequence, four were C<->T transitions, five were A<->G transitions and two were A<->T transversions (Table 6.1).

The two haplotypes defining the KZN north coast and Richards Bay samples (haplotypes 1 and 2) differed at a single base (Table 6.1). Frequency differences at this position were observed for the presence of an adenine or guanine residue, such that 73% of Richards Bay sample displayed the G residue, defining haplotype 1, while 82% of the north coast sample displayed the A residue, characterising haplotype 2. A guanine residue at position 115 appeared to be fixed for the KZN south coast and East Cape samples, defining haplotype 3. A fourth haplotype, characterised by an adenine at position 169 was found in a single KZN south coast sample (Table 6.1). Average nucleotide diversity (Nei, 1987) among South African humpback dolphins was estimated at 0.13 %.

Analysis of 10 samples from Hong Kong revealed a high level of nucleotide variation in the portion of the control region sequenced, with 15 transition substitutions (A<->G = 13; C<->T = 2) defining four haplotypes. Average nucleotide diversity for humpback dolphins from the Hong Kong coast was estimated at

0.70%. Thirteen fixed nucleotide differences distinguished Hong Kong humpback dolphins from South African samples, two of which were transversion substitutions. Six of these sites, including the transversions, were shared with the Maputo Bay sample.

6.3.2 DNA divergence

Genetic distance ranged from 0.25 % to 0.77 % among South African haplotypes (Table 6.2) with an average of 0.51 %. Taking into account the level of diversity within each group, divergence estimates ranged from 0.12 % to 0.64 % between groups within South Africa, with an average divergence of 0.14 % for the whole sample.

Genetic distance ranged from 1.03 % to 3.61 % (Table 6.2) with an average of 2.2% among all Hong Kong haplotypes. This translates to an average sequence divergence (Nei, 1987) of 1.0 % among haplotypes. Sequence divergence was estimated at 2.8 % between South African and Maputo Bay haplotypes and after compensating for sequence polymorphism within either region, a large intraspecific divergence of 5.6 % was estimated between South African and Hong Kong haplotypes.

6.3.3 Phylogenetic analysis

Only three substitutions defined the four haplotypes found in the South African sample. However, with the inclusion of Hong Kong samples in the phylogenetic analysis, 32 of the 400 sequenced bases were variable, 24 of which were parsimony informative. Maximum parsimony analysis using the branch and bound search option, generated a single tree of 59 steps (Figure 6.1), which supports the division of southern African and Hong Kong humpback dolphins into two reciprocally monophyletic clades. The two south coast/East Cape haplotypes formed the terminal nodes of the southern African clade, while the Maputo Bay haplotype was placed basal to the South African haplotypes. The same branching pattern was produced for the maximum likelihood tree (Figure 6.2), determined from 287 trees under the Kimura 2-parameter model (Kimura, 1980).

The Hong Kong and South African clades are supported by, respectively 97 % and 100 % of the 1000 bootstrap replications in the neighbour-joining tree (Figure 6.3). Furthermore, well-supported internal nodes demonstrate good confidence in the branching order of South African haplotypes. However, the neighbour-joining tree differs from the maximum parsimony and maximum likelihood trees in the placement of the Maputo Bay haplotype in a position basal to both the South African and Hong Kong clades. This may be a result of two transversion substitutions, common to both Maputo Bay and Hong Kong haplotypes having increased the gamma distances between the Maputo Bay and South African haplotypes, placing the former in a more basal position than is seen with parsimony and likelihood methods.

6.3.4 Microsatellite variation and allele frequency differences

Moderate to low microsatellite variability was observed for humpback dolphins. A maximum of 50% of the loci examined for South African humpback dolphins were polymorphic, with individuals from the KZN south coast and Cape coast displaying variation in only one-third of the loci tested. However, 66.7 % of the same loci were polymorphic in Hong Kong samples (Table 6.3). A maximum of five alleles were scored for locus 199/200, four of which were found in South African animals and three in the Hong Kong sample. Only two of the five alleles were common to dolphins from both regions. Of the four alleles resolved for locus 464/465, only two were found in South African samples. Locus 409/470 was monomorphic in

South African samples, but yielded two alleles in Hong Kong samples (Table 6.3; Fig. 6.4d). Allelic diversity was low, with an average number of alleles per locus of 1.8 for the South African sample and 2.2 for Hong Kong dolphins. The average number of alleles per polymorphic locus (A_p) was estimated at 2.7 and 3.0, respectively, indicating that variation was low even within polymorphic loci. Average observed heterozygosity was 0.263 for South African samples and 0.495 for Hong Kong samples.

Locus 415/416 showed little difference in allele frequency, both within the South African sample and between the South African and Hong Kong samples (Fig. 6.4a). Notable differences in frequency occurred for loci 464/465 and 199/200 between the South African and Hong Kong samples. Alleles 146, 154 (locus 464/465) and 192 (locus 199/200) were absent from South African humpback dolphins, while alleles 154 and 158 (locus 199/200) were not scored in Hong Kong dolphins. Within the South African sample, the same loci displayed allele frequency differences. The south coast sample revealed only two alleles at locus 199/200, one of which occurred at a frequency of 0.917. Alleles 158, 166, 170 (locus 199/200), 106 and 110 (locus 464/465) occurred at similar frequencies in the Richards Bay and north coast groups, while allele 110 was fixed at locus 464/465 in the south coast dolphins.

For all loci, the tendency was toward amplification of shorter alleles in South African samples. Hong Kong dolphins displayed similar frequencies for alleles scored for locus 415/416, while the tendency was toward amplification of longer alleles for loci 199/200 and 409/470.

Table 6.1. Three variable sites characterised four haplotypes in South African humpback dolphins. A fifth haplotype was detected in a single sample from Maputo Bay. Four haplotypes characterised the Hong Kong sample. Dots indicate sites which are identical to those in the consensus sequence. KwaZulu-Natal south coast and East Cape samples are combined as a single locality (S.C./E.C.). Nucleotide positions are given from 5' to 3' on the mtDNA heavy strand (similarity block E to tRNA^{PRO}in Southern *et al.* (1988)).

Haplotype #	Nucleotide position	Locality				
	111111 1111122233 3333333333 33 1119001111 3556915601 1244555566 78 0128020345 1259804492 3435123712 71	Richards Bay	North coast	S.C./E.C.	Maputo Bay	Hong Kong
1	AGAATGATGA TGTAGTGCGA TACTTAGGAG AT	11	2			
2	........A.	4	9			
3	.........G ...G......			5		
4	.........G			1		
5	....CA.CA. ....TCATAA.. G.				1	
6	G.GGCAG.A. CAC..C.T.G C...AT.AGA G.					3
7	...GCAG.A. CAC..C.T.G C.T.ATAA.. G.					1
8	G.G.CAG.A. C.CGACATAG CG..AT.AGA G.					2
9	GAGGCAG.A. CAC..C.T.G C.T.ATAA.. GC					4

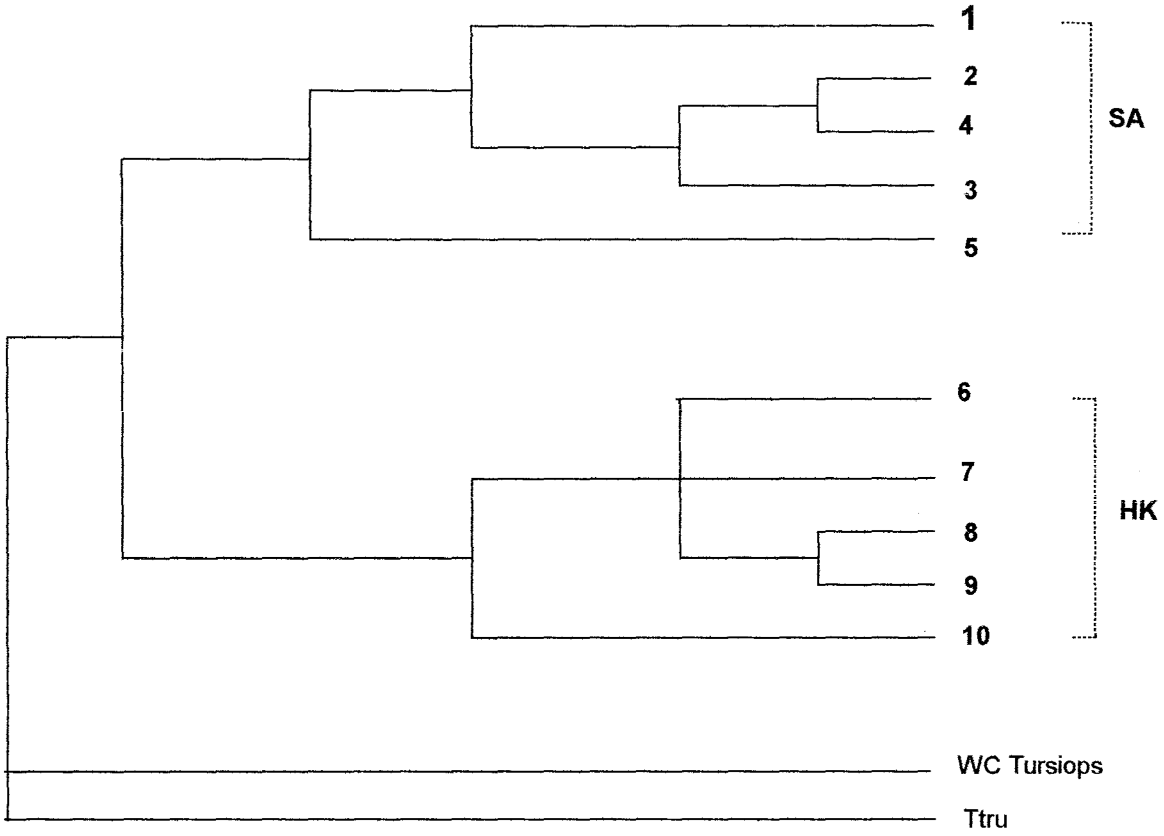


Figure 6.1 Maximum parsimony analysis (MEGA; Kumar *et al.*, 1993) of humpback dolphin haplotypes generated a single tree of 59 steps. The tree is rooted to two offshore bottlenose dolphin sequences. Haplotype numbers correspond with those in Table 6.1. Sequences from South African west coast *Tursiops* (WC Tursiops) and US Atlantic *Tursiops* (Ttru) comprise the outgroup.

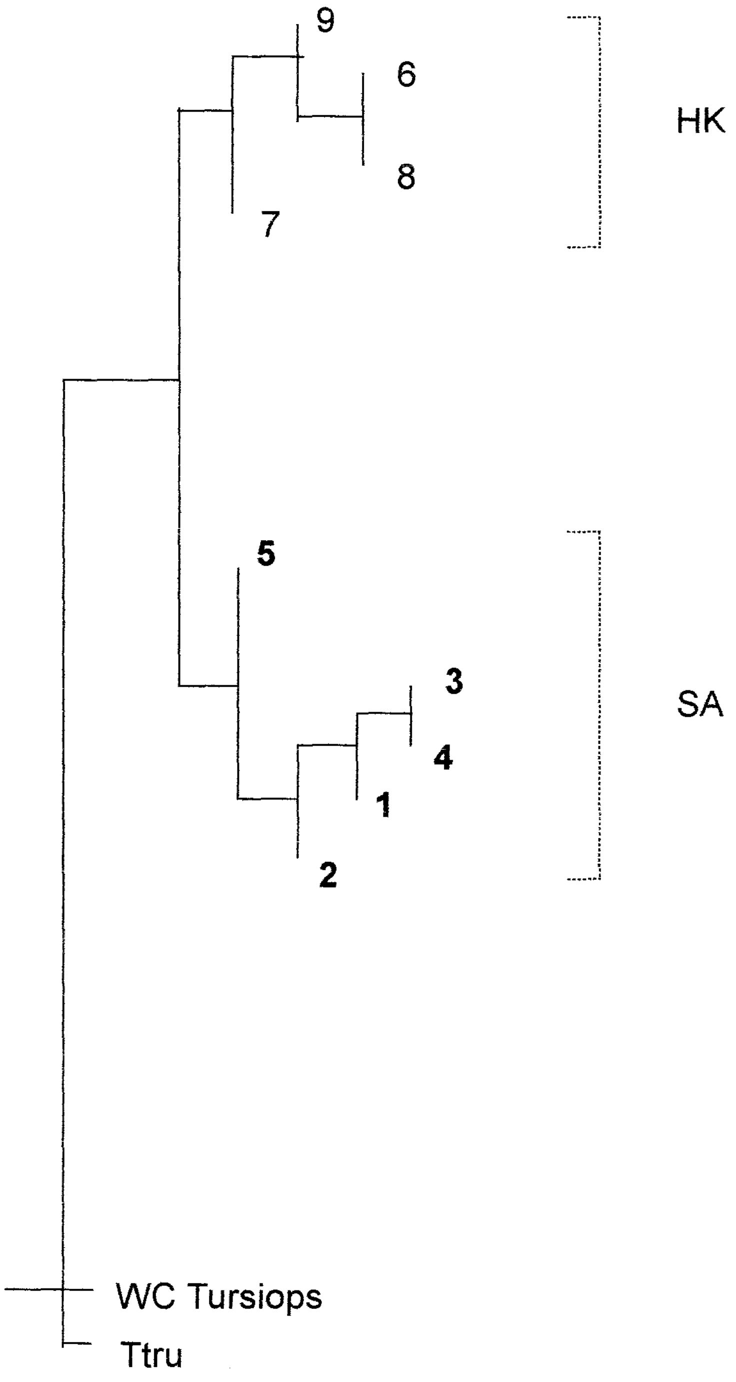


Figure 6.2 Maximum likelihood of 287 trees generated using PHYLIP 3.5 (Felsenstein, 1993). Log likelihood is -954.52. Haplotype names and numbers correspond with those in Table 6.1. Outgroups are as described in Fig. 6.1.

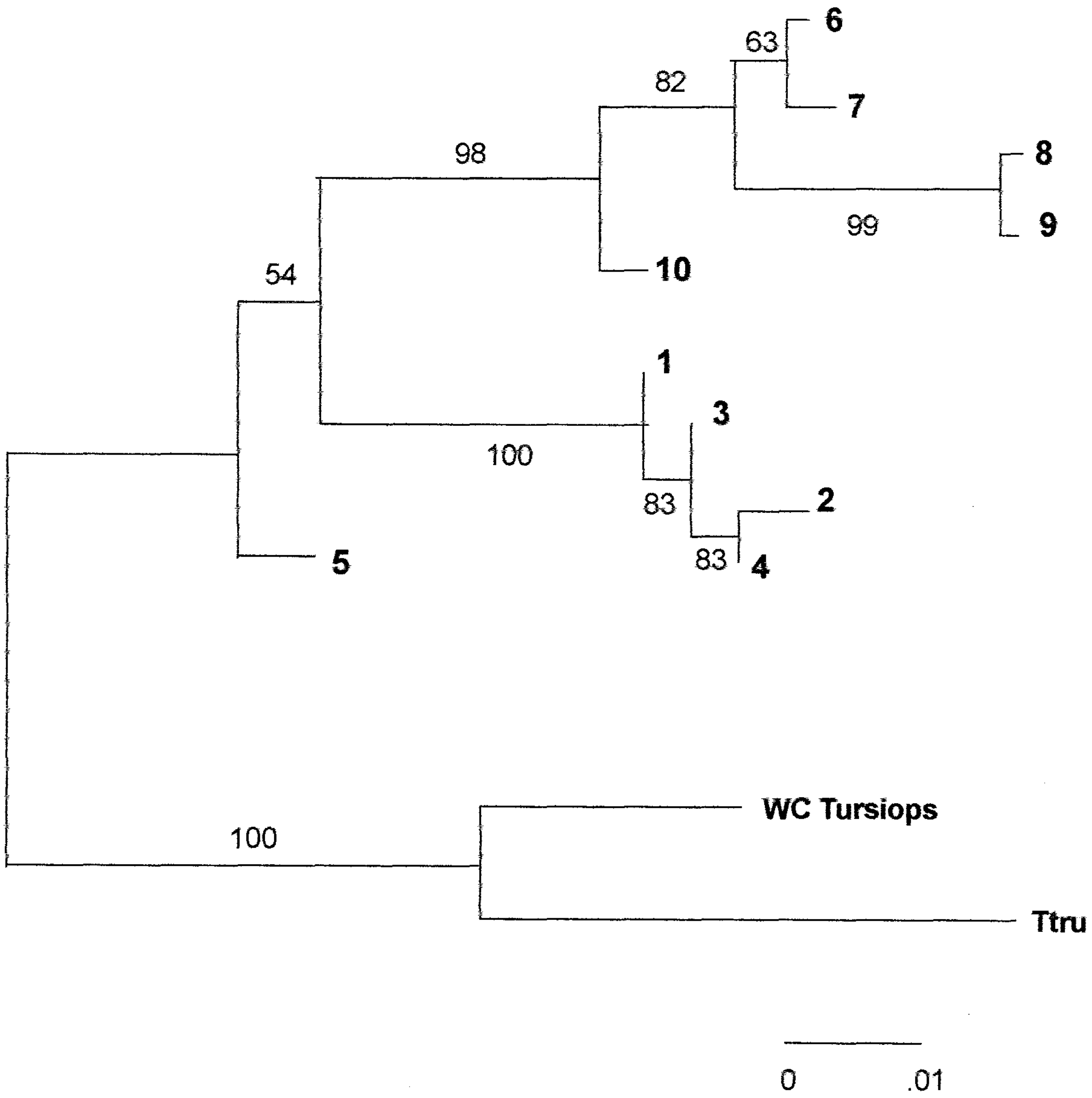


Figure 6.3 Majority rule consensus tree generated from 1000 bootstrap replications using the neighbour-joining algorithm in MEGA (Kumar *et al.*, 1993). Numbers at the nodes represent bootstrap values in percentages. The branches are scaled in terms of distance units. Haplotype numbers correspond with those in Table 6.1. Outgroups are as described in Fig. 6.1.

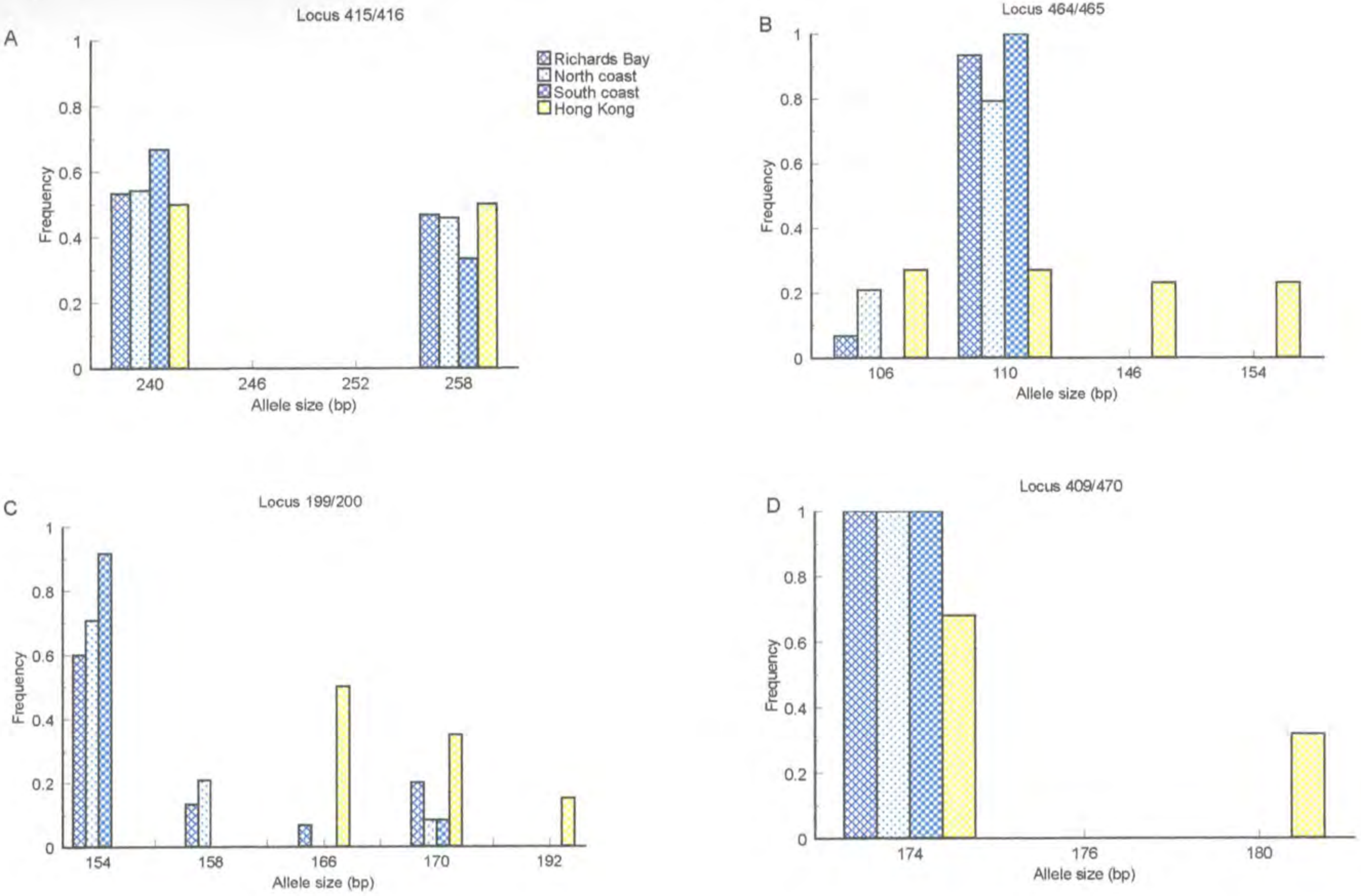


Figure 6.4 Allele frequency histograms for polymorphic microsatellite loci in South African and Hong Kong humpback dolphins.

Table 6.2. Nucleotide variation in nine humpback dolphin mtDNA haplotypes. Genetic distance is expressed in percentages as gamma distances ($\alpha = 0.5$) calculated using the Kimura 2-parameter model (Kimura, 1980) (above diagonal). The number of nucleotide substitutions is partitioned into transitions / transversions (below diagonal). Haplotype numbers correspond with those in Table 6. 1.

	1	2	3	4	5	6	7	8	9
1		0.25	0.51	0.25	2.96	5.79	5.13	6.84	6.48
2	1/0		0.77	0.51	2.67	5.46	4.8	6.48	6.14
3	2/0	3/0		0.25	3.56	6.48	5.79	6.84	7.19
4	1/0	2/0	1/0		3.26	6.14	5.46	7.19	6.84
5	9/2	8/2	11/2	10/2		4.87	3.00	5.88	4.23
6	18/2	17/2	20/2	19/2	17/0		1.57	1.85	1.57
7	16/2	15/2	18/2	17/2	11/0	6/0		3.61	1.03
8	21/2	20/2	21/2	22/2	20/0	7/0	13/0		3.61
9	20/2	19/2	22/2	21/2	15/0	6/0	4/0	13/0	

Table 6.3. Allele sizes for four polymorphic microsatellite loci in humpback dolphins from South Africa and Hong Kong. Dashes indicate the absence of an allele from the population.

Locus	Size of alleles (bp)	Frequency of alleles	
		South Africa	Hong Kong
415/416	240	0.59	0.5
	258	0.41	0.5
464/465	106	0.11	0.27
	110	0.89	0.27
	146	-	0.23
	154	-	0.23
199/200	154	0.69	-
	158	0.14	-
	166	0.03	0.50
	170	0.14	0.30
	192	-	0.15
409/470	174	1.0	0.68
	180	-	0.32

Table 6.4. Genetic variability at six microsatellite loci in humpback dolphins. Inbreeding coefficient (F_{IS}) is given by 1- (H_O / H_E) (see section 3.2.4.2). Data from microsatellite analysis are compared with preliminary allozyme data (Smith, 1990). None of the groups indicated significant inbreeding. Standard errors are shown in parentheses.

	Microsatellites		Allozymes
	South Africa N=33	Hong Kong N=11	South Africa N=16
Mean number of alleles per locus (A)	1.8 (0.5)	2.2 (0.5)	1.07 (0.05)
Mean number of alleles per polymorphic locus (A_p)	2.7 (0.9)	3.0 (0.8)	2.0 (0.0)
Percentage of loci polymorphic (P)	50	66.7	13.6
Mean direct count heterozygosity (H_O)	0.263 (0.146)	0.495 (0.191)	0.033 (0.020)
Mean expected heterozygosity (H_E)	0.196 (0.098)	0.405 (0.135)	0.032 (0.018)
Inbreeding coefficient (F_{IS})	-0.342	-0.222	-0.031
χ^2 test for significant inbreeding	$\chi^2 = 1.25$	$\chi^2 = 0.296$	$\chi^2 = 0.005$

Table 6.5. Chi-squared (χ^2) deviations from Hardy-Weinberg expectations in polymorphic microsatellite markers resolved for humpback dolphins; df = degrees of freedom, p = statistical significance. Asterisks denote significance at $\alpha < 0.05$. Hardy-Weinberg estimations were not determined for locus 464 for the south coast sample as it displayed fixation for a single allele. Values in parentheses indicate χ^2 estimates after pooling of rare alleles (section 3.2.4.2).

	Richards Bay			North coast			South coast			Hong Kong			
	415	464	199	415	464	199	415	464	199	415	464	199	409
χ^2	10.6	0.04	10.1	7.76	0.64	7.92	1.07	-	0.00	4.00	31.0	6.29	2.00
						(2.41)					(1.250)		
df	1	1	6	1	1	3	1	-	1	1	6	3	1
						(1)					(1)		
p	0.001*	0.85	0.119	0.005 *	0.423	0.048 *	0.301	-	1.000	0.046 *	0.000 *	0.134	0.157
						(0.121)					(0.264)		

6.3.5 Deviation from Hardy-Weinberg equilibrium

Deviations from expectation were seen in all three polymorphic loci (Table 6.5). The Richards Bay and north coast groups showed a significant excess in the number of heterozygotes scored for locus 415/416. A marginally significant deviation from equilibrium was indicated at locus 199/200 in the north coast group; however, the χ^2 test with pooling revealed that this was a result of the presence of rare alleles. The south coast sample was found to be in equilibrium for loci 415/416 and 199/200, but was fixed for allele 110 at locus 464/465. The Hong Kong group displayed expected genotype frequencies at loci 199/200 and 409/470. The χ^2 test with pooling found no significant deviation from equilibrium at locus 464/465. Although high inbreeding coefficients were noted within both regions (Table 6.4), neither was significantly different from zero.

6.3.6 Comparison with allozyme variability

As expected, the mean number of alleles per locus was higher in microsatellite markers than in allozyme loci (Table 6.4). Mean expected heterozygosity was approximately six times greater in the six microsatellite loci studied than in 19 putative allozyme loci screened (Smith, 1990).

6.3.7 RAPD variation

Of the 20 random primers screened, three failed to amplify the DNA in 80% of the samples, 11 yielded inconsistent amplification products and the remaining six primers generated monomorphic fingerprints in agarose gels. The products from the latter six primers were separated on 10% nondenaturing polyacrylamide gels in an effort to detect polymorphic fragments. Large intragel variation and complex fingerprints, generated by the amplification of up to 30 bands, resulted in a large variance in the scoring of band patterns both by eye and using the GelCompar^{TM} version 4.0 software. The large number of smaller fragments (<200 bp) and bands of light intensity resolved with vertical polyacrylamide gels were probably a result of sheared DNA present in many of the extracts, which, instead of aiding in the resolution of the fingerprints, merely confounded the scoring of true RAPD alleles. As a result of this and the unexpectedly low RAPD variation seen with agarose gels, no further RAPD analysis was conducted for humpback dolphins.

6.4 DISCUSSION

6.4.1 MtDNA sequence variation and phylogeny reconstruction

Mitochondrial DNA nucleotide diversity in South African humpback dolphins was lower than analogous values reported for Hong Kong humpback dolphins and other marine mammals (refer to Table 3.6), but similar to the value determined for bottlenose dolphins from the same stretch of coastline (Chapter 3). Furthermore, it lies close to the values reported for the narwhal (Palsbøll *et al.*, 1994) and the cheetah (Menotti-Raymond and O'Brien, 1993), the former suspected to have originated from a small founder population and the latter proposed to be the result of an ancient bottleneck in the population. In contrast, humpback dolphins from Hong Kong, however, revealed a similar level of nucleotide diversity to large, outbred populations of California sea lions (Maldonado *et al.*, 1995) and harbour porpoise (Walton, 1997).

The intraspecific sequence divergence of 5.6 % between South African and Hong Kong humpback dolphins is considerably larger than many analogous values noted for other marine mammal populations (Table 3.6). Furthermore, it lies close to the intergeneric divergence between the harbour porpoise and Dall's porpoise (6.05 %, Rosel, 1992). However, high intraspecific divergences are not uncommon among vicariant populations of marine mammals. Maldonado *et al.* (1995) found differences in excess of 5 % between control region haplotypes of California sea lions from the Pacific coast and the Gulf of California, while a sequence divergence of 5.4 % was observed in minke whales from Antarctica and the western north Pacific (Wada *et al.*, 1991).

The large intraspecific sequence divergence and reciprocal monophyly of South African and Hong Kong humpback dolphin haplotypes indicates a long history of isolation between the two populations. An approximate time since divergence can be calculated for the two humpback dolphin populations, using the equation r = d/2T (Kimura, 1980; Wilson *et al.*, 1985) and the estimated rate of divergence of 0.5 - 1.0 % per MY (Hoelzel *et al.*, 1991b, Baker *et al.*, 1993). A divergence estimate of 5.6 %, suggests that a common ancestor of South African and Hong Kong humpback dolphins occurred 2.8 - 5.6 million years ago. Division within the delphinidae occurred between four and 10 million years ago (Hoelzel *et al.*, 1991b; Ohland *et al.*, 1995), suggesting that the South African and Hong Kong humpback dolphin mtDNA lineages may have diverged early in the radiation of the species. However, the rate of substitution in the control region appears to vary considerably (Wang *et al.*, 1996) and given a slightly higher rate of substitution, the values of divergence will become more recent. Furthermore, there is currently no supporting evidence for this having occurred. Further investigation into the phylogeny of *Sousa* is underway to attempt to resolve the currently disputed taxonomy of the genus.

With the exception of the placement of the Maputo Bay sample in the neighbour-joining tree (Fig.6.3), all three methods used to infer the phylogeny of humpback dolphins from control region data, display concordance regarding the topology of the consensus tree, suggesting that the relationship indicated by phylogenetic analysis may approximate the true phylogeny of the haplotypes examined (Kim, 1993). Short branch lengths joining South African taxa reflect few differences among haplotypes. As suggested for bottlenose dolphins, this may be a result of either a recent founder or bottleneck event, with limited time for the subsequent radiation of haplotypes (refer to section 6.4.4).

6.4.2 Variation at nuclear loci

Although variation in microsatellite markers of South African samples was higher than that in allozyme markers, it was still lower than variation in the microsatellite markers of Hong Kong dolphins. While four of the six loci tested polymorphic in Hong Kong dolphins, only three were polymorphic in South African samples and estimates of allelic diversity and heterozygosity were also higher in the former.

The finding that a maximum of four loci displayed any polymorphism across the two species of dolphin in this study, suggests that a lack of species-specificity may well have contributed towards the low variation detected (see section 3.4.2. for a detailed discussion). However, differences in levels of variation in both mitochondrial and nuclear loci between the two populations of humpback dolphin support the opinion that genetic diversity in South African humpback dolphins is low. Furthermore, it is suspected that the Hong Kong sample used in this study is not representative of the entire population as samples were collected opportunistically from stranded animals. Thus, the level of molecular variation in Hong Kong dolphins may well have been under-estimated. Given that completely different alleles were found for animals from the north and south of Lantau Island (see Chapter 7), it is probable that more intensive sampling along the coastal regions inhabited by humpback dolphins would uncover additional alleles. By contrast, if current population estimates are correct, the South African sample used in this study, comprises approximately 16.5 % of the total number of dolphins occurring off KwaZulu-Natal. Additional sampling off KZN would probably not increase the allelic diversity estimates; however, sampling of humpback dolphins in Algoa Bay and regions to the south west, may resolve additional alleles not found on the KZN coast.

6.4.3 Effective population size

Mitochondrial DNA nucleotide diversity can be used to determine the long term effective female population size, given the nucleotide substitution rate of 0.5 - 1.0 % per million years (Hoelzel *et al.*, 1991b; Baker *et al.*, 1993; but see section 3.4.4.1). Long term $N_{e(f)}$ for South African humpback dolphins is estimated at 13 100 - 26 200 individuals. This the value is similar to that determined for bottlenose dolphins in this study (Chapter 3); however, given the significant structure seen in the mtDNA of South African humpback dolphins (Chapter 7), it probably represents an inflated estimate of long term $N_{e(f)}$ (Waples, 1991). By contrast, mtDNA nucleotide diversity in Hong Kong dolphins implies an $N_{e(f)}$ of 70 200 - 140 400, which is comparable to the $N_{e(f)}$ estimated from published studies of striped dolphins and is greater than the estimate reported for minke whales (Hoelzel and Dover, 1991b; and see section 3.4.4.1).

Avise *et al.* (1988) reported census populations considerably larger than $N_{e(f)}$ in the redwing blackbird, a species with high gene flow and low fecundity. They suggested that the variance between N and $N_{e(f)}$ was a result of recent extensive colonisation and expansion of the species. The inverse is presented here for South African humpback dolphins and the tendency is to suspect the relationship between N and long term $N_{e(f)}$ represents a recent reduction in census population.

Using the temporal method proposed by Waples (1989, 1991) and the stratification of generations desribed in section 3.4.4.2, a current N_e of 38 was estimated for KZN humpback dolphins. This value is, however, probably under-estimated, due both to the restricted movement among proposed home ranges (see Chapter 7) and to the lack of temporal discreteness of the generations (Waples, 1991). A demographic estimate of short term N_e, proposed to lie within the range of 0.2 to 0.5 times the size of the census population (Crow and Kimura, 1970; Mace and Lande, 1991), can be estimated at between 40 and 100 individuals, assuming a census population of 200 humpback dolphins off the KZN coast. This does not differ substantially from the N_e estimated from genetic data, suggesting that the level of genetic variation noted for KZN humpback dolphins is approximately the level expected in a population that size. However, if the demographic N_e for the entire south east African coast is considered, the two values would differ substantially. Although no accurate census is available for the region south of KZN, Karczmarski (1996) proposed a population size of 200-400 individuals in Algoa Bay and the adjacent south west regions alone. Thus, assuming a total population of 1000 humpback dolphins in the region covered by this study, a demographic N_e of 200 - 500 animals is expected. In this case, the discrepancy between temporal genetic N_e and the demographic N_e for the whole coast is likely the result of insufficient sampling of regions south of KZN. Given the suggestion of a larger population in these regions, additional sampling may uncover greater genetic variation, thus elevating the genetic N_e, though, given the level of variation indicated by the KZN sample, this increase is not expected to be large.

6.4.4 Evolutionary history of South African humpback dolphins

The large difference in nucleotide diversity noted between South African and Hong Kong humpback dolphins suggests that, either the Hong Kong population is considerably older than that off South Africa, suggesting that the latter originated recently as the result of a founder event, or that the South African population has been subjected to a decline in diversity as a result of a population bottleneck in its recent history. Given that similar levels of diversity have been found in bottlenose and humpback dolphins off the coast of South Africa, it is probable that the evolution of both species has been shaped by similar events.

The estimated sequence divergence among mtDNA control region haplotypes in South African humpback dolphins dates homogeneity of the region sequenced to 70 000 to 140 000 years BP. As with bottlenose dolphins, this approximates the onset of the last glacial age of the Pleistocene, when the sea level dropped beyond the continental shelf off the south east African coast (Heydorn *et al.*, 1978; Deacon and Lancaster, 1988 and Chapter 4). Although it is possible that sympatric dolphin species could colonise a coastal region at the same time, it is more likely that existing populations would be simultaneously affected by a stochastic event which would reduce their numbers, causing a reciprocal decline in genetic variability. Thus, a population bottleneck appears to have been the more likely cause of the current level of variability observed in both species. A similar hypothesis has been proposed for the low genetic diversity observed in the cheetah (Menotti-Raymond and O'Brien, 1993). The authors suggest that the current level of variation is the result of a dramatic population bottleneck suffered by the cheetah during a time when a notable extinction of large vertebrates occurred (op cit.).

The large sequence divergence between Maputo Bay and South African haplotypes may reflect the level of pre-glacial diversity in humpback dolphins along the length of the east and south east African coast. It is possible that inshore dolphins in Maputo Bay and further north were not as severely affected by a decline in sea level as the continental shelf widens considerably at the bay. Furthermore, shallow waters around the islands comprising the Bazaruto archipelago, off the coast of Mozambique, may have provided sufficient inshore refugia for coastal dolphins. However, this remains mere speculation in the absence of samples from regions north of South Africa. To test adequately, the hypothesis of a bottleneck having occurred along the south east African coast, the level of diversity as well as the phylogenetic relationships of both species of inshore dolphins from Mozambique and further north requires thorough investigation.

6.5 CONCLUSIONS

It appears that the South African humpback dolphin population is also characterised by historically low variability. In the absence of additional pressure, the current level of variation would probably not hinder their resilience to selective and stochastic processes. However, exploitation constitutes a dramatic environmental change to marine mammals that have evolved at or near their carrying capacity, subjecting them to selective forces fundamentally different from many of those under the influences of which they evolved (Estes, 1979).

Continued removal of demographically important or reproductively active individuals from the population, such as occurs on the KZN coast, reduces the current effective population size and ultimately, results in further reduction in the level of variation. This, in turn, affects the immediate response of the population to selective forces (Nei *et al.*, 1975, Allendorf, 1986). Furthermore, dolphin populations are particularly sensitive to depletion through exploitation and recover very slowly once exploitation has ceased (Estes, 1979). In a small population such as that on the KZN coast, a slow recovery rate, coupled with the potential for depressed fitness, in the presence of continued exploitation may eventually result in local extinction.

CHAPTER 7

POPULATION STRUCTURE AND GENE FLOW IN S.E. AFRICAN HUMPBACK DOLPHINS

7.1 INTRODUCTION

The degree of genetic differentiation among natural populations is an important factor in deciding upon the appropriate geographic scale at which to pursue conservation and management efforts (Milligan *et al.*, 1994). In the marine environment, defining population subdivisions often requires knowledge of parameters other than geographic boundaries. For marine mammals particularly, the extent of differentiation varies both spatially and temporally where reproductive barriers are defined by social and behavioural factors (Dizon *et al.*, 1992).

Population structure and genetic differentiation are the products of the combined effects of mutation, genetic drift due to limited population size and local adaptation favoured by natural selection. Gene flow, in the form of dispersal of gametes, individuals or populations will challenge the tendency towards differentiation (Slatkin, 1987). Together, these forces determine the phylogeography of a species. Gene flow within and among populations is viewed as the result of an interplay between the intrinsic dispersal capabilities of the species and extrinsic barriers to that dispersal (Ball *et al.*, 1988).

Avise *et al.* (1987) recognised three fundamental levels of phylogenetically-supported population structure. The first comprises species which are capable of wide dispersal, occur in ranges lacking barriers to gene flow and consequently, show limited genetic structure. The second level recognises varying degrees of genetic distance between subdivisions, but comprises species which show phylogenetically separate geographic clades in their range. The greatest level of differentiation is characterised by species in which monophyletic groups can be distinguished as a result of long term restriction to gene flow. In this case, isolated populations will evolve, with time, to a state of reciprocal monophyly.

7.1.1 Population structure in South African humpback dolphins

The degree of reproductive interaction among schools, groups or populations (as defined in section 2.2.3) identified along the coasts of KwaZulu-Natal and the eastern and southern Cape, is one of the most influential parameters by which the need for special conservation and management of the humpback dolphin will be measured. Presently, dispersal data for the species on the south east African coast are highly speculative. Recent field studies in KZN and the East Cape suggest that the humpback dolphin displays varying degrees of fidelity to the area in which it is found. Karczmarski (1996) found little evidence of residency in humpback dolphins frequenting a study site along the East Cape coast, while Saayman and Tayler (1979) reported seeing humpback dolphins throughout the year in another East Cape locality. They also reported several resightings of individuals during a three year study period. Durham (1995), working along the KZN coast, described a high degree of resighting in certain areas, while humpback dolphins appeared to be absent from large sections of the coast. Similarly, Natal Sharks Board sighting data and analyses of incidental captures (Cockcroft, 1990b) point to areas of greater abundance. All of these data suggest that the extent of movement of the humpback dolphin in relation to its mobility is variable throughout its range and is probably determined by a series of complex environmental and behavioural factors.

7.1.2 Objectives

In order to conserve the humpback dolphin population in KZN, it is necessary to determine whether they and animals from adjacent regions, comprise a single population. In order to determine the extent of subdivision and gene flow among geographically-determined subdivisions outlined in Table 6.1, the following indicators of population differentiation were estimated:

1) differentiation in a portion of the mtDNA D-loop was determined at several hierarchical levels using AMOVA (Excoffier et al., 1992),

2) differentiation in microsatellite DNA was determined at several hierarchical levels using R_{ST} (Slatkin, 1995) and AMOVA,

3) the degree to which spatial differentiation accounts for total population diversity in microsatellite DNA was determined, using F_{ST} (θ; Weir and Cockerham, 1984),

4) gene flow estimates were calculated from ϕ_{ST}, R_{ST} and F_{ST}, to determine the level of gene exchange among geographical areas.

7.2 MATERIALS AND METHODS

7.2.1 Sample collection

Samples were collected as described in sections 2.2.1.2 and 2.2.2.2.

7.2.2 DNA analysis

DNA extraction, amplification, sequencing and electrophoresis were conducted as described in sections 2.3 to 2.5. Microsatellite loci were amplified and resolved as outlined in section 2.6. A molecular determination of the gender of skin biopsy samples and stranded samples was conducted using the PCR (section 2.8).

7.2.3 Nomenclature and group designation

The geographical subdivision used for testing genetic structure was defined by capture and sighting data collected over an approximate 10-year period. For the purpose of this study, the KZN coast was divided into a northern and southern region with the division occurring at Durban. The northern region, termed the Tugela Bank, was further divided into the 'north coast' and 'Richards Bay'. Samples taken from the eastern and southern Cape were included with KZN south coast samples for two reasons: 1) only a single sample was acquired from each of Algoa Bay and False Bay, and 2) sequencing of a portion of the control region revealed identical haplotypes (see Table 6.1) in samples from these regions and the KZN south coast.

Hong Kong samples collected from localities in the New Territories and the northern coast of Lantau Island were analysed as a 'north Lantau' sample. Those collected at localities along the southern coast of Lantau Island and Cheung Chau and Soko Islands were analysed as a 'south Lantau' group.

7.2.4 Data analysis

7.2.4.2 Population structure

7.2.4.2.1 Estimation of population structure from mtDNA

The AMOVA (Excoffier *et al.*, 1992) was used to determine the degree of differentiation within and among designated populations and groups, as described in section 5.2.4.1.1. A matrix of squared Euclidean distances was constructed for all pairwise comparisons of the nine mitochondrial haplotypes resolved for Southern African and Hong Kong humpback dolphins. Population differentiation was assessed for various groupings as suggested by phylogenetic analysis and geographical distribution.

7.2.4.2.2 Estimation of population structure from microsatellite DNA

Variation in allele frequencies was used to calculate population heterogeneity in microsatellite DNA. An estimation of the degree to which spatial differentiation accounts for total population diversity was expressed as the jack-knifed mean of F_{ST} (θ; Weir and Cockerham, 1984). In addition, the AMOVA (Michalakis and Excoffier, 1996) was used to obtain ϕ_{ST}, an analogue of Slatkin's R_{ST} (Slatkin, 1995). These values were determined as described in section 5.2.4.1.2.

7.2.4.3 Gene flow

7.2.4.3.1 Estimation of gene flow from mtDNA

The number of female migrants per generation, Nm_f, was calculated from ϕ_{ST} using the equation modified from Takahata and Palumbi (1985), as described in section 5.2.4.2.1.

7.2.4.3.2 Estimation of gene flow from microsatellite DNA

Average F_{ST} (θ) over all loci and the R_{ST} analogue, ϕ_{ST} were used to determine gene flow in microsatellite DNA, as described in section 5.2.4.2.2.

7.3 RESULTS

7.3.1 Geographic distribution of mtDNA control region haplotypes

South African humpback dolphin mtDNA haplotypes displayed clear geographical segregation. Of the four haplotypes resolved, two were found exclusively in samples taken north of Durban, while the remaining two haplotypes were found only in samples taken south of Durban, as far as False Bay. Haplotype 1 predominated in the Richards Bay group (73 %), while the majority (82 %) of animals sampled from the north coast displayed haplotype 2 (Table 6.1; Fig. 7.1). Five of six south coast samples were scored for haplotype 3, while the remaining sample characterised a fourth haplotype, which differed by a single transition.

In contrast to the pattern noted for South African dolphins, there was no geographic concordance among closely-related haplotypes in the Hong Kong population. In fact, all haplotypes were distributed erratically throughout the sample area.

7.3.2 Geographic distribution of microsatellite DNA genotypes

Genotype frequency differences were detected for the three polymorphic loci in South African samples. Richards Bay and the north coast both displayed 106/110 heterozygotes for locus 464/465, while all samples taken from the south coast were homozygous for allele 110 (Fig. 7.2a). The 154/154 homozygote scored for locus 199/200 showed a southward increase in frequency, while the number of genotypes declined from four in the most northern grouping to two in the most southern samples (Fig. 7.2b). The two northern groups displayed the 240 bp homozygote for locus 415/416 in almost identical low frequency, while one third of the south coast sample was resolved for that genotype.

Clear geographical differences were seen in samples taken from the north and south of Lantau Island, Hong Kong. Figure 7.3 represents the distribution of polymorphic genotypes. The three localities situated to the north of Lantau Island and one in the New Territories landmass (see Fig. 2.2b) displayed, exclusively, the 146/154 genotype for locus 464/465 and the 166/170 genotype for locus 199/200. Localities to the south of Lantau Island, including the Soko and Cheung Chau Islands, displayed the 106/110 genotype for locus 464/465 and the 170/192 genotype for locus 199/200. A single Cheung Chau sample was homozygous for allele 166 at locus 199/200. The homozygous 180 genotype for locus 409/470 was only found in two samples from south of Lantau, while the homozygous 174 genotype predominated in the northern sample. The genotype of locus 415/416 could not be resolved for all but three of the samples. A single anomalous sample, which had a genotype profile identical to that of a Cheung Chau sample, was located at a site far north, on the New Territories landmass. Although its discovery may be cause for interesting discussion, it is not within the scope of this thesis to investigate it further.

7.3.3 Genetic differentiation

7.3.3.1 Genetic differentiation estimated from mtDNA

As expected, the South African and Hong Kong populations displayed significant mitochondrial DNA differentiation, with 90 % of genetic variance resulting from differences between the two populations (Table 7.1). Further subdivision exists within the South African population. The geographical distribution of control region haplotypes suggested differentiation of mitochondrial DNA between sampling areas on the Tugela Bank and those to the south (Fig. 7.4). The AMOVA confirmed that significant heterogeneity exists between the two regions (ϕ_{ST} =

0.796, $p < 0.0005$). Moreover, when the Tugela Bank was further divided into the Richards Bay and north coast study groups, the degree of heterogeneity declined only marginally ($\phi_{ST} = 0.748$, $p < 0.0010$), with as much as 41 % of the differentiation attributed to variance between the two groups. Differentiation was neither influenced by the seasonal distribution of haplotypes nor by the regional occurrence of males or females.

The clear geographical differentiation seen in the South African humpback dolphin mtDNA was not evident in that of the Hong Kong population. Division of the samples into those taken from north of Lantau Island and those taken from areas south of it, revealed that all of the genetic variance is accounted for by differences within the two subdivisions (Table 7.1).

7.3.3.2 Genetic differentiation estimated from microsatellite DNA

As with mitochondrial control region data, significant microsatellite DNA differentiation was evident between the South African and Hong Kong populations. Significant differentiation between the Tugela Bank and the rest of the coast was implied by the R_{ST} value, however, the F_{ST} estimate for that grouping was not significant (Table 7.2). None of the remainder of the groupings investigated for the South African sample showed significant structure in the microsatellite loci studied (Table 7.2), despite the suggestion of geographical differences in genotype frequencies (Figure 7.2). Similarly, fractional seasonal variance was observed and no differentiation was detected between males and females.

In contrast to the limted structure found in the South African population, the Hong Kong population was significantly subdivided around Lantau Island. More than 11% of the genetic variance observed in the microsatellites tested, was attributed to differences between samples collected from the north and south of the island. Both the R_{ST} analogue, ϕ_{ST} and F_{ST} (Weir and Cockerham, 1984) were significantly greater than zero when tested using the χ^2 test for genetic heterogeneity (Workman and Niswander, 1970).

7.3.4 Gene flow and migration

7.3.4.1 Gene flow estimated from mtDNA

The number of migrants per generation was estimated between the South African and Hong Kong populations as a relative indication of the degree of gene flow between reproductively isolated populations. As expected, the estimated number of migrant females between the two populations was less than 0.1. Even within the South African population, fewer than one migrant female per generation was exchanged between adjacent localities (Table 7.3). Dividing the coast into a northern and a southern group, with the division at Durban, showed a level of gene flow between the two groups of fewer than 0.2 females per generation. Even the slight overlap in group-specific haplotypes between Richards Bay and the north coast appeared to be insufficient to prevent genetic drift-associated differentiation between the two areas (Nm_f = 0.69), despite their geographical proximity.

7.3.4.2 Gene flow estimated from microsatellite DNA

High levels of gene flow were observed for the four polymorphic microsatellite loci studied. Migration appeared to be highest between Richards Bay and the KZN north coast, with a migration rate of more than 60 individuals per generation and lowest between these areas and the south coast, with 2-6 migrants per generation (Table 7.3). Contrary to a lack of mtDNA differentiation, marked microsatellite differentiation was detected in the Hong Kong humpback dolphin population. Gene flow between the regions north and south of Lantau Island appeared to be severely restricted, with an exchange rate of fewer than one individual per generation.

7.3.5 Mother-calf relationships

Two suspected mother-calf pairs were analysed for mitochondrial DNA type. One pair, incidentally captured on the KZN north coast, displayed identical mitochondrial haplotypes. The second pair was taken in shark nets at Richards Bay. The suspected mother from the second pair displayed haplotype 1, the more common haplotype seen in the Richards Bay sample, while the calf captured with her, possessed haplotype 2, occurring predominantly in the north coast sample, confirming that the two animals were not maternally related.

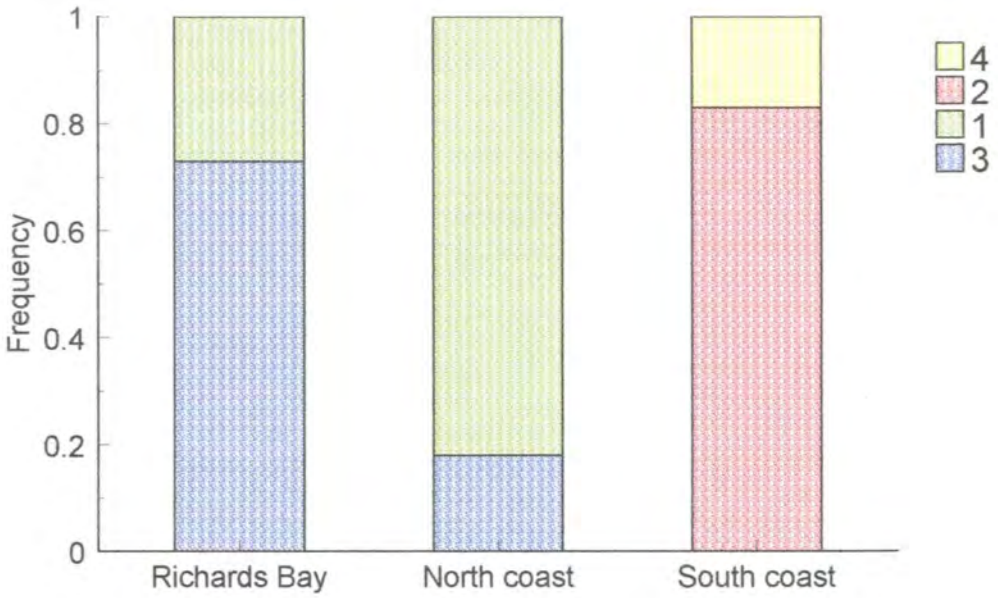


Figure 7.1 Mitochondrial DNA haplotype frequencies found in South African humpback dolphins. The Tugela Bank is divided into Richards Bay and the KZN north coast, while 'south coast' refers to all samples originating south of Durban. Haplotype numbers correspond with those in Table 6.1.

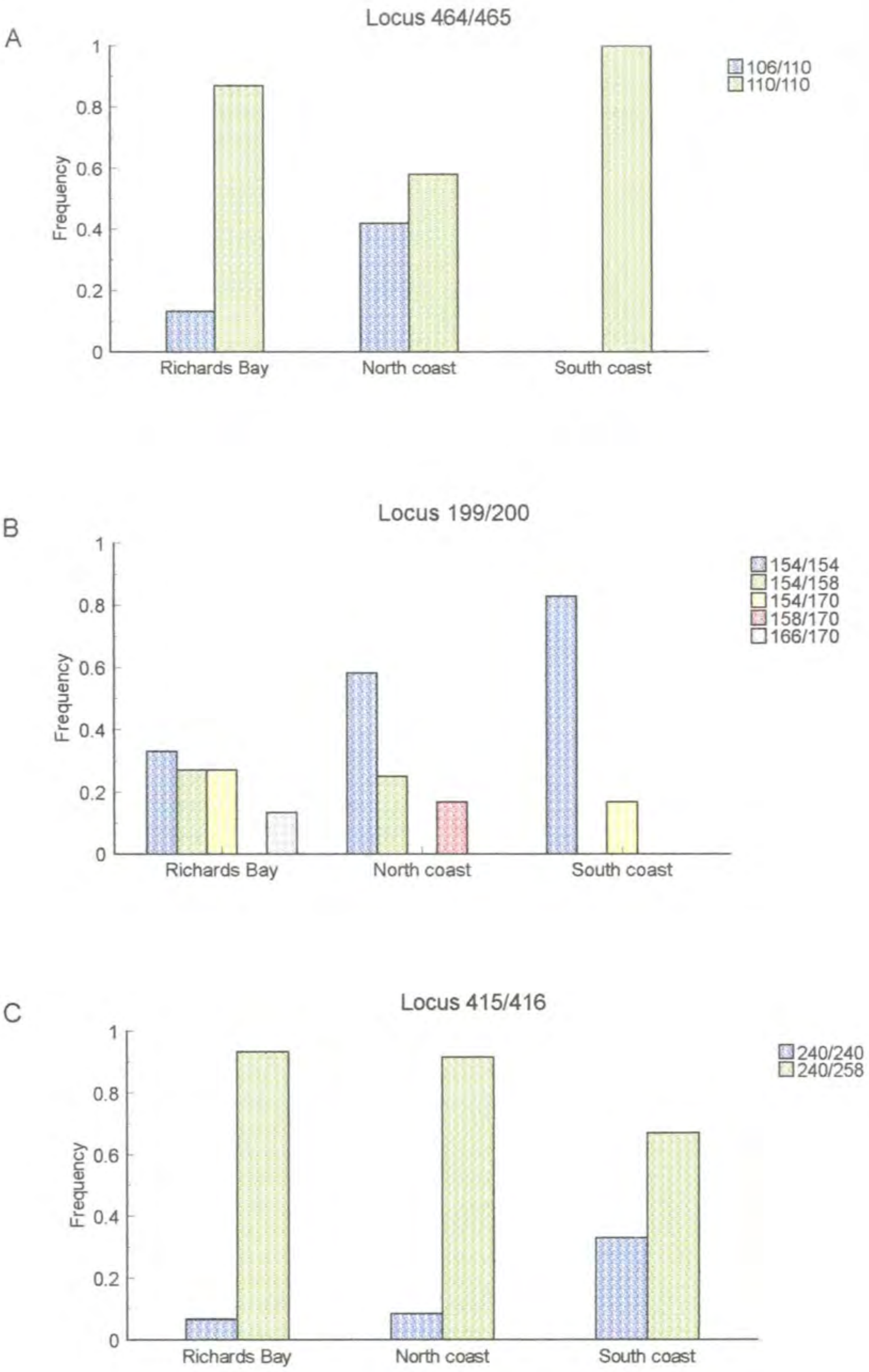


Figure 7.2 Microsatellite genotype frequencies for three polymorphic loci in South African humpback dolphins.

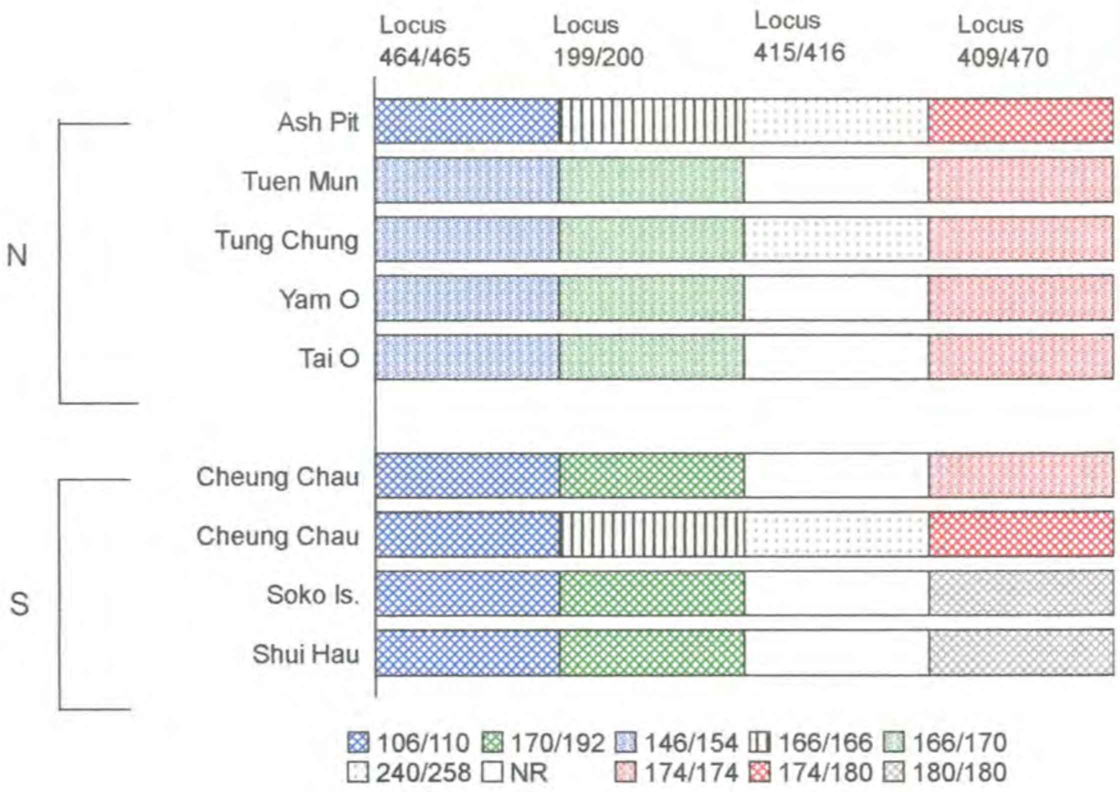


Figure 7.3 Microsatellite genotype frequencies for polymorphic loci in Hong Kong humpback dolphins. The top five localities represent north of Lantau Island and the lower four represent sampling sites to the south of the island. Refer to Fig. 2.2b for location of sampling sites. NR = not resolved.

Table 7.1 Partitioning of mtDNA genetic variance in humpback dolphins by AMOVA. AP is the among-populations component of variance, AG is the among-groups component of variance, WP is the within-populations component of variance and AP/WG defines the variance among populations within groups. SA is the South African population (excluding Maputo Bay) and HK refers to the Hong Kong population.

Groups	Populations	Variance Component	% total variance	ϕ statistics	Permutations	p value
Whole sample	S A	AP	90.07	$_{ST}$ 0.901	2 000	< 0.0005
	H K	WP	9.93			
SA	Tugela Bank	AP	79.63	$_{ST}$ 0.796	2 000	< 0.0005
	Rest of coast	WP	20.37			
S A	Richards Bay	AP	74.84	$_{ST}$ 0.748	2 000	< 0.0010
	North coast	WP	25.16			
	South coast					
KZN north coast	Richards Bay	AP	41.86	$_{ST}$ 0.419	2 000	< 0.0010
	North coast	WP	58.14			
Richards Bay	Male	AG	76.48	$_{CT}$ 0.765	2 000	< 0.0010
North coast	Female	AP/WG	-3.64	$_{SC}$ -0.155		0.7390
South coast		WP	27.16	$_{ST}$ 0.728		< 0.0010
Richards Bay	Summer	AP	73.48	$_{CT}$ 0.735	2 000	< 0.0010
North coast	Winter	WP	-2.48	$_{SC}$ -0.093		0.7103
South coast			29.00	$_{ST}$ 0.710		< 0.0010
H K	N. Lantau	AP	-19.91	$_{ST}$ -0.199	2 000	0.7510
	S. Lantau	WP	119.91			

Table 7.2 Partitioning of microsatellite allele frequency variance in South African and Hong Kong humpback dolphins. Variance components estimated by AMOVA are as described in Table 7.1. Significance testing of both the R_{ST} analogue, ϕ_{ST} , and F_{ST} was performed following Workman and Niswander (1970) and is presented as χ^2 values. Three asterisks denotes significance to p<0.001, a double asterisk denotes significance to p<0.025 and a single asterisk denotes significance to p<0.05.

Groups	**Populations**	**Variance components**	**% total variance**	**ϕ statistic (R_{ST} analogue)**	**χ^2 value (df)**	**F_{ST}**	**χ^2 value (df)**
Whole sample	South Africa Hong Kong	AG WP	14.12 85.88	$_{ST}$ 0.141	24.25*** (1)	0.345	29.67*** (1)
SA	Tugela Bank Rest of coast	AP WP	4.71 95.29	$_{ST}$ 0.047	6.204** (1)	0.041	2.706 (1)
S A	Richards Bay North coast South coast	AP WP	1.28 98.72	$_{ST}$ 0.013	1.716 (2)	0.023	1.518 (2)
S A	Male Female	AP WP	-0.60 100.60	$_{ST}$ -0.006	0.792 (1)	-0.013	0.858 (1)
S A	Summer Winter	AP WP	1.98 98.02	$_{ST}$ 0.020	2.640 (1)	0.027	1.782 (1)
H K	N. Lantau S. Lantau	AP WP	11.49 88.51	$_{ST}$ 0.115	4.600* (1)	0.341	6.820*** (1)

Table 7.3 Gene flow in humpback dolphins from South Africa and Hong Kong, represented as the number of migrants per generation, estimated from mtDNA and microsatellite differentiation.

Populations	Population structure and gene flow estimates						
	Mitochondrial DNA		Nuclear microsatellite DNA				
	ϕ_{ST}	Nm_f	F_{ST}	Nm	R_{ST}	M_R	d_s
Hong Kong - South Africa	0.901	0.055	0.345	0.474	0.141	0.762	2
Richards Bay - North coast	0.419	0.693	0.003	83.08	0.002	62.38	2
Tugela bank - Rest of coast	0.796	0.128	0.041	5.850	0.047	2.530	2
N. Lantau - S. Lantau	-0.199	-	0.341	0.483	0.115	0.962	2

7.4 DISCUSSION

7.4.1 Maternal genome differentiation

Social cetacean populations may behave as small, isolated or semi-isolated demographic units, rather than as one large group of freely interbreeding individuals (McMillan and Bermingham, 1996). Although their vast capacity for dispersal would allow occasional gene flow between units, random genetic drift may, nonetheless, act independently within each unit, resulting in local differentiation of mtDNA lineages (Slatkin, 1987). Genetic evidence from this study has suggested that South African humpback dolphins form several demographic units displaying mtDNA differentiation, with occasional, if any, maternal gene flow. Unexpectedly, this differentiation corresponded well with the geographical groupings into which the study population was divided. Analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) supported the inferences made by mtDNA haplotype frequency data, revealing significant heterogeneity both within the Tugela Bank and between it and the rest of the east coast (Table 7.1), suggesting that female humpback dolphins on the South African east coast are strictly philopatric within ranges varying from a few to several hundred kilometres. Furthermore, a large sequence divergence suggests that humpback dolphins from Maputo Bay and South Africa may be reproductively isolated. With gene flow of fewer than one female per generation among the three areas within South Africa (Table 7.3), it is possible that mtDNA differentiation has occurred by random genetic drift due to protracted isolation of humpback dolphins in each geographical region (Slatkin, 1987). Although sampling error introduced by estimating gene flow from a single haploid locus may bias N*m* (Bowen *et al.*, 1992), the gene flow estimates derived from mtDNA are nonetheless useful for drawing qualitative conclusions about the dispersal of female humpback dolphins.

Cetacean populations display varying levels of mtDNA structure, despite enjoying similar potentials for dispersal. Baker *et al.* (1990, 1994) found significant genetic subdivision among three major oceanic populations of humpback whales (*Megaptera novaeangliae*). Despite an almost unlimited capacity for dispersal and in the absence of extrinsic barriers, lineages within ocean basins were further segregated into subpopulations, demonstrating the species' substantial fidelity to migratory destinations (*op cit.*, 1994). In contrast, Dall's porpoise (*Phocoenoides dalli*) displayed limited phylogeographic distinctiveness among mtDNA haplotypes, implying moderate to high levels of gene flow among Pacific and Bering Sea populations (McMillan and Bermingham, 1996), while striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea displayed no evidence of population structure, implying panmixia, at least along the Spanish coast and neighbouring islands (Garcia-Martinez *et al.*, 1995).

Similarly, individuals sampled from the Hong Kong humpback dolphin population gave no indication of regional geographic differentiation of mtDNA haplotypes. This incongruity within the species may be an indication that the animals sampled and the haplotypes resolved from the Hong Kong population, originated from a larger, variable population, covering a range which extended further than the sample area. In this way, the large, protected bay habitat may provide a focal area for schools or populations from coastal areas to the east and west. Thus, studying a population within a limited portion of its range, may have resulted in an over-estimation of its homogeneity and an absence of genetic structure in the Hong Kong humpback dolphin population may merely be a reflection of population structure over a much larger area.

7.4.2 Nuclear genome differentiation

An analysis of molecular variance using R_{ST} confirmed the variation in microsatellite genotype frequency which differentiates the Tugela Bank groups from the south coast samples. However, Weir and Cockerham's (1984) F_{ST} value indicated that spatial differentiation did not account for a significant degree of the total population diversity (Table 7.2). The lack of correspondence between the two estimates may be a result of the type of mutation believed to occur in microsatellite alleles (Valdes *et al.*, 1994). The calculation of F_{ST} does not account for the possibility of forward-backward mutation and the potential to return to historical allelic states. As a result, population differentiation would be under-estimated (Allen *et al.*, 1996). The R_{ST} estimate (Slatkin, 1995) accounts for the microsatellite mutation process and is expected to produce a less biased calculation of genetic differentiation (Allen *et al.*, 1996).

Significant differentiation in the nuclear DNA of humpback dolphins from the Tugela Bank and the southern region suggests that all members of the population may show fidelity to defined area, within which females may be strictly philopatric. The significant segregation of microsatellite genotypes displayed by dolphins from the north and south of Lantau Island, Hong Kong, further indicates the tendency of the species towards local philopatry. Assuming that the South African population's current genetic assemblage is the result of a recent genetic bottleneck (Chapter 6), it is likely that, with sufficient time, further differentiation may occur to reveal greater structure in the nuclear loci.

7.4.3 Phylogeographic population structure

Heirarchical classification schemes have been proposed to describe the population genetic structure and phylogeography of species requiring conservation and management (Avise *et al.* 1987; Avise, 1989, Dizon *et al.*, 1992). These range from populations which are both genetically and geographically distinct to those which show extensive gene exchange corresponding with an absence of subdivision by geographical barriers. Avise *et al.* (1987; Avise, 1989a) described category III species as displaying continuous mtDNA phylogenies with few mutations between phylogenetically adjacent clades, but which are confined to geographically disjunct regions within the range of the species. They are also characterised as having limited gene flow between populations in the absence of physical barriers to dispersal. Dizon *et al.* (1992) applied the same criteria to the definition of marine mammal stocks, adding that geographically distinct mtDNA assemblages develop regionally as a result of local selective pressure. From the results of this study, humpback dolphins from the Tugela Bank and the southern region may be regarded as isolated or semi-isolated subpopulations within the South African population. Similarly, the South African and Maputo Bay populations may represent isolated populations within the southern African coastal region. Thus, with an overall population structure conforming to an "island' or "stepping stone" model (Hartl, 1988), the humpback dolphin in south east Africa may be classified as a category III species (Avise *et al.*, 1987; Dizon *et al.*, 1992).

Avise *et al.* (1987) could find few examples of populations satisfying the description of category II populations, in which genetic discontinuity and large divergence values are found between closely related genomes which are not geographically subdivided. The Hong Kong humpback dolphin population may represent a category II population, with several mitochondrial haplotypes diverging by as much as 3.6% (Table 6.2), in spite of a lack of obvious geographical partitioning. Insufficient samples do not permit the grouping of haplotypes into composite mitochondrial assemblages, thus it may only be assumed that the haplotypes resolved represent only a portion of those existing in the population as a whole. Hoelzel and Dover (1991a) described sympatric populations of killer whale (*Orcinus orca*) off Vancouver as category II populations when they found substantial genetic differentiation between animals utilising different prey sources. Similarly, two sympatric forms of the common dolphin (*Delphinus delphis*) occurring in the north Pacific, displayed fixed mtDNA nucleotide differences as well as morphological variation (Rosel *et al.*, 1994) indicating reproductive isolation between the two forms despite their geographical continuity.

The implications of classifying the phylogeographic relationships of cetacean populations extend far beyond academic worth. Conservation of vulnerable species and management of exploited populations require the synthesis of all genetic and distributional data with life history parameters in order to accurately define comprehensive and pragmatic policies (Chapter 8).

7.4.4 Population structure and gene flow in south east African humpback dolphins

Speculation on the degree of residence versus long-range movement of humpback dolphins on the south east African coast arose from field research (Saayman and Tayler, 1973, 1979) and photo-identification data (Durham, 1995; Karczmarski, 1996). Combining results from field studies with genetic data strengthens existing knowledge of the range and movement of the species and permits a more comprehensive interpretation of the social and geographic factors that influence these patterns.

7.4.4.1 Social structure and local site fidelity

The extent of co-operative calf care among female humpback dolphins is unknown. A mature female and a young calf caught simultaneously in a shark net at Richards Bay and assumed to be a mother-calf pair, were shown to have different mtDNA types. This may support field evidence that allomaternal care occurs in this species. Humpback dolphin females and calves in Algoa Bay were seen several times forming temporary associations (Karczmarski, 1996), while young calves in Plettenberg Bay were reported to have spent prolonged periods with different adult animals (Saayman and Tayler, 1973).

Significant differentiation in mtDNA assemblages between the two localities within the Tugela Bank region suggests that female humpback dolphins are strictly philopatric within a limited range of their habitat. Such local site fidelity has been noted in coastal bottlenose dolphins (Duffield and Wells, 1991; Duffield *et al.*, 1989) and has been accompanied by a predictable structuring of social groups with respect to age and gender (Wells and Scott, 1990; Wursig and Harris, 1990). Of the individuals resighted in both Algoa Bay (East Cape; Karczmarski, 1996) and Richards Bay (Tugela Bank; Durham 1995) the majority were females. Karczmarski (1996) proposed that females may display fidelity to a small area while caring for young calves. He suggested that the demands made on females during lactation require that they remain in a resource-rich, shallow, protected area. This may render them available for resighting more frequently than other age-sex classes.

Frequent resighting of solitary males at localities 80 to 120 km apart (Durham, 1995; Karczmarski, 1996), suggested that males move extensively within large areas. Furthermore, several large males were seen interacting with different schools throughout the study period in Plettenberg Bay (Saayman and Tayler, 1979). This supports the suggestion that males move freely among schools, representing the principle vectors of genetic exchange in a similar way to that noted for male bottlenose dolphins in Sarasota Bay, Florida (Duffield and Wells, 1991; Duffield *et al.*, 1989; Scott *et al.*, 1990).

7.4.4.2 Movement within home ranges

Although males appear to disperse more widely than females, it appears that movement is restricted to defined home ranges. Although nuclear gene flow between the Tugela Bank and the rest of the coast appears to be sufficient to prevent isolation by drift, it is approximately 20 times lower than that occurring within the Tugela Bank (Table 7.3), suggesting that all humpback dolphins may observe a degree of fidelity to a locale representing a home range, in this case, the Tugela Bank.

Field studies have also indicated that humpback dolphins display a general tendency to be found in certain localities with a degree of regularity. Sighting frequencies and incidents of capture in shark nets are low in the KZN south coast region, implying that humpback dolphin abundance in this region is low. In contrast, the Tugela Bank region accounts for 87 % of humpback dolphin sightings and more than 65 % of the incidental captures (Cockcroft, 1990b; Durham, 1995). This would suggest that the Tugela Bank constitutes a home range within which males and perhaps to a very limited extent, females, rove among schools. Further south, Saayman and Tayler (1979) reported seeing humpback dolphins throughout the year in the vicinity of Robbeberg in Plettenberg Bay (Fig. 7.4), at least two of which remained in the area for the duration of the three-year study period.

Although long range movements of up to 120 km have been observed for male humpback dolphins (section 7.4.4.1 above), all of the resightings occurred within either the Tugela Bank or Algoa Bay/St. Francis Bay region (Durham, 1995; Karczmarski, 1996). Resighting data provided no evidence of movement between the Tugela Bank and the KZN south coast (Durham, 1995), nor between the Tugela Bank and the East Cape (Durham, 1995; Karczmarski, 1996). This suggests that the Tugela Bank, extending from the Tugela River at least as far as Richards Bay, constitutes a home range within which fidelity to local sites is observed by females, while males roam continually throughout the range. The extent of this home range is likely influenced by the geography of the coastline. The narrow continental shelf extending from southwards from Durban to Algoa Bay would not be suitable for resident inshore dolphins, thus restricting humpback dolphins to a region where the shelf is sufficiently wide to ensure a shallow inshore area (Fig. 7.4).

Defining home ranges for humpback dolphins on the KZN south coast is not possible in the absence of additional samples. However, data from field studies (Saayman and Tayler, 1979; Karczmarski, 1995) suggest that the home ranges of humpback dolphins off the East Cape may comprise several adjacent bays. Field studies have recorded observations of humpback dolphins year-round in Algoa Bay, St Francis Bay, Jeffreys Bay and Plettenberg Bay, located consecutively along the East Cape coast (Saayman and Tayler, 1973; 1979, Karczmarski, 1996). Furthermore, resightings of individuals occurred within 48 hours between adjacent bays, indicating that humpback dolphins in the East Cape readily range distances of 80-100km between bays (Karczmarski, 1996). Low resighting frequencies and long lapses between resightings, as well as the continual identification of new individuals (Karczmarski, 1996) suggests that Algoa Bay constitutes only a portion of the home range of the dolphins studied. Furthermore, temporal fluctuations in abundance in Algoa Bay (Karczmarski, 1996) complemented those observed in Plettenberg Bay (Saayman and Tayler, 1979), suggesting that humpback dolphins may range between the two localities, covering a distance of up to 260km. The high degree of immigration and emigration noted by Karczmarski (1995) in Algoa Bay may be a result of the bay representing the most northern extent of a home range extending further south.

Coupled with the abovementioned field data, identical mtDNA haplotypes and similar microsatellite allele frequencies noted for samples from Algoa Bay and False Bay (Fig. 7.4), suggests that home ranges in the eastern and southern Cape may overlap. The wider continental shelf and series of contiguous bays extending from Algoa Bay to False Bay would provide an uninterrupted inshore habitat for humpback dolphins, facilitating gene flow along the length of the East Cape coast.

7.4.4.3 Regional distribution

The clear division between humpback dolphins sampled on the KZN north coast and those sampled further south was an unexpected result. Although humpback dolphin distribution has been reported to extend the length of the south east coast of South Africa (Ross, 1984), there appear to be areas which are clearly low in abundance and others which support higher densities of dolphins (Durham, 1995; Karczmarski, 1996). The available sighting data provided insufficient information to attempt to match breaks in distribution with geographical features of the coast. However, genetic data from this study suggest that the apparent isolation of East Cape dolphins from Tugela bank dolphins may be explained by both social and geographic factors.

From Ponta do Ouro (Fig. 7.4) in the north to Algoa Bay in the south, the South African east coast is relatively straight with little protection from prevailing weather or current conditions. Furthermore, it is characterised by a very narrow continental shelf over most of its length (Heydorn *et al.*, 1978; Schumann, 1987), such that the coastal ocean area is extremely limited in places. North of St.Lucia (Fig. 7.4), the extremely narrow shelf gives way to steep canyons (Heydorn *et al.*, 1978) and swift water and sediment movement occurs as the Agulhas Current passes close to the shore (Schumann, 1987). As the humpback dolphin is a strictly nearshore, shallow water species, often restricted to estuaries (Cockcroft, 1991), deltas (Zbinden *et al.*, 1977) and shallow inlets (Saayman and Tayler, 1973, 1979; Corkeron, 1990) the coast between Mozambique and St.Lucia may not provide a suitable habitat. South of St. Lucia, extending to just south of Durban, the shelf widens to more than 45km and is characterised by shallow, undulating topography (Heydorn *et al.*, 1978). It is here that the Tugela Bank is found, a shallow muddy belt of terrigenous deposits originating at the Tugela delta (op cit.). Several large rivers, including the Tugela River, open into this region of the coastline, providing a rich estuarine habitat for many of the humpback dolphin's prey species (Cockcroft and Ross, 1983). It is likely that the Tugela Bank represents the most suitable habitat for the species in KwaZulu-Natal. Between Durban and Port Alfred (Fig. 7.4), where the shelf is narrowest and steepest the 50m isobath lies very close to the shore (op cit.) and the effects of the Agulhas Current are pronounced (Schumann, 1987). South of Durban, both sightings and catch rates (Durham, 1995) are significantly lower (Cockcroft, 1990b; Durham, 1995) and there have been few reports of humpback dolphins along the coast between southern KZN and Algoa Bay (Cockcroft personal communication). The shelf widens again around Algoa Bay and extends about 50km offshore, south of Cape Recife (Schumann, 1987). The coast is convoluted into numerous bays and this, together with the reduced effects of the Agulhas Current, results in nearshore conditions and an increase in the abundance of humpback dolphins in the inshore zone. The suggestion, then, is that the stretches of coastline from Ponto do Ouro to St. Lucia and from the Tugela Bank to Algoa Bay probably represent ecological deterrents to dispersal, resulting in extremely limited, if any, gene flow and subsequently, genetic differentiation between humpback dolphins in the remaining parts of the coast.

However, having noted this, identical mtDNA haplotypes and similar microsatellite genotype frequencies were found among samples from localities such as the KZN south coast and False Bay. Given the proposed tendency of the species towards philopatry (sections 7.4.4.1 and 7.4.4.2) and the unsuitable coastal conditions spanning these regions, it is unlikely that regular gene flow occurs over the 1300 km separating them.

Although humpback dolphins have not been reported to follow the annual 'sardine run' *en masse*, as occurs with bottlenose dolphins and common dolphins (*Delphinus delphis*) (Peddemors, 1995), larger-than-usual schools have been observed off the Transkei and KZN south coast during the winter months (V.M. Peddemors, personal communication). Thus, it is not inconceivable that individuals or small schools could follow the migration of pilchards and predatory fish, many of which constitute important prey species (Barros and Cockcroft, 1991). In the absence of either field or genetic evidence to support the occupation of home ranges extending north of Algoa Bay into the KZN south coast, an incidental long range shift in distribution in response to the annual biotic migration may be an alternative explanation for the continuity of mtDNA haplotypes and microsatellite genotype frequencies. In opposition to this is the fact that none of the animals identified in Algoa Bay (Karczmarski, 1996) was resighted in KZN (Durham, 1995) and vice versa. However, given the low sighting frequency on the KZN south coast and the relatively short study periods in which animals were identified, it is unlikely that matches would have occurred. Moreover, if humpback dolphins were resident on the KZN south coast, it would be expected that common nuclear genotype frequencies or mtDNA haplotypes would be found between them and the Tugela Bank residents, given the geographical proximity of the two regions.

In support, fewer than 10 humpback dolphins were caught in shark nets on the entire KZN south coast over an eight year period, while more than 60 animals were caught on the Tugela Bank (Cockcroft, 1990b). Durham (1995) suggested that humpback dolphins which are caught in shark nets on the KZN south coast are more likely to be transient, given the lack of suitable habitat and the low sighting frequency along that stretch of coast. More important is the fact that all of the incidental shark net captures of humpback dolphins within an eight-year period occurred at the most southern beaches on the KZN coast. None occurred north of Ifafa, which marks the northern limit of the 'sardine run' (Cockcroft, 1990b; see section 5.4.5), suggesting that humpback dolphins captured incidentally on the KZN south coast may well be migrants from the southern or eastern Cape, following the sardines and associated biota.

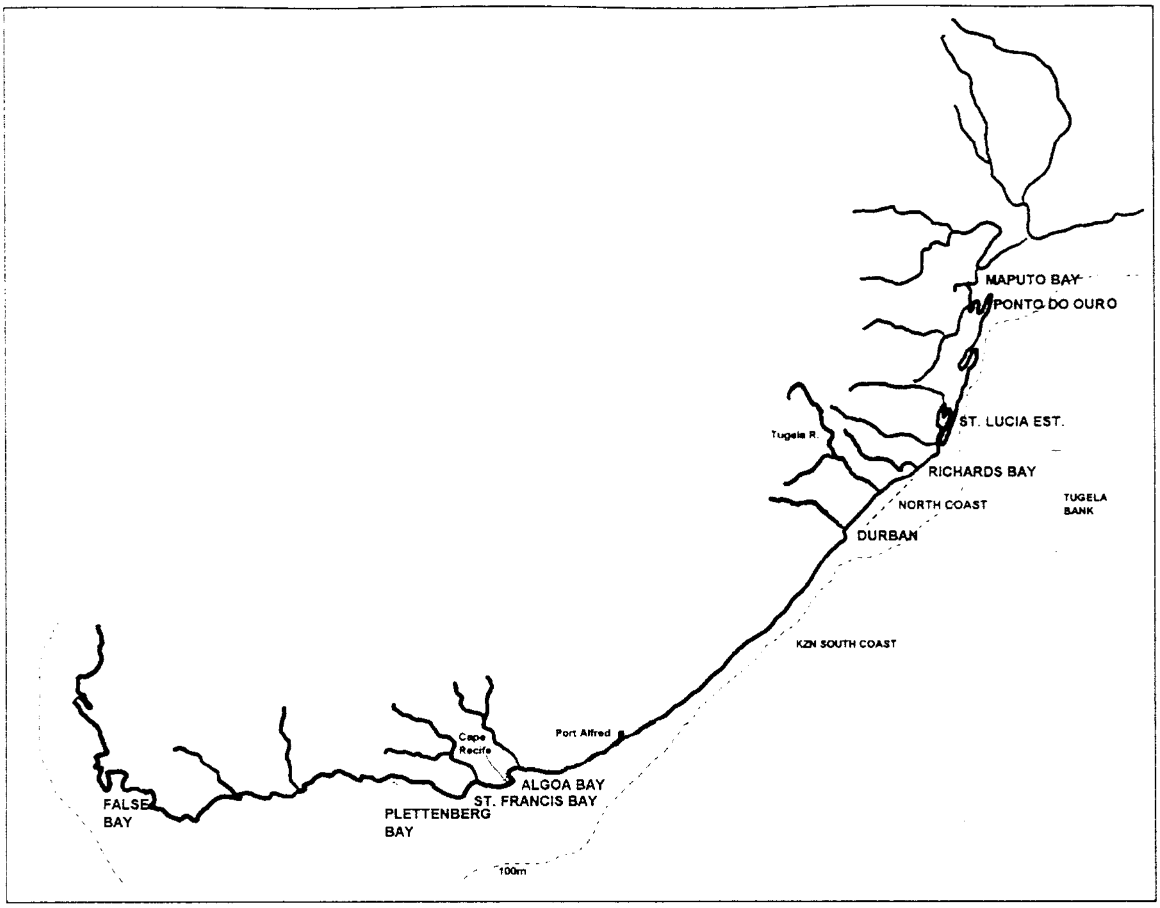


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Figure 7.4 The geographical characteristics of the south east African coast as they pertain to the distribution of humpback dolphins. Maputo Bay, the Tugela Bank and the southern Cape are characterised by several large estuaries which open onto a wide continental shelf. The KZN south coast and the region between the St. Lucia Estuary and Ponto do Ouro comprise an exposed coastline with a narrow continental shelf. The shelf is indicated by the 100m isobath.

7.5 CONCLUSIONS

The findings of this study suggest that the South African humpback dolphin population comprises at least two discrete stocks, the first occurring on the Tugela Bank off the KZN north coast and the second occurring in the eastern and southern Cape. Significant population subdivision was evident within the Tugela Bank region, although there appears to be sufficient nuclear gene flow between localities to prevent isolation. Insufficient samples precluded investigation into further subdivision within the Cape subpopulation, although, given the degree of structure found in the Tugela Bank subpopulation, it is possible that genetic structuring occurs over the 1000km of coastline spanning the eastern and southern Cape. Humpback dolphins sampled on the KZN south coast may either comprise residents of large overlapping home ranges extending from the East Cape into southern KZN, or, more likely, are incidental long range migrants from the East Cape.

Females on the Tugela Bank appear to be strictly philopatric, favouring either Richards Bay or the group of estuaries adjacent to the Tugela River. Insufficient samples from the southern regions prevented further investigation into female philopatry, nevertheless, the available genetic data, coupled with field data suggest that females may show fidelity to a larger range in the eastern and southern Cape. This is likely associated with the abundance and distribution of prey, water depth and calving periods. As a species, the humpback dolphin appears to display degrees of residence to different localities. Furthermore, fidelity may not be limited to females and calves and once again, may be strongly influenced by habitat conditions.

A comparison of the different types of population structure seen in South African and Hong Kong humpback dolphin populations, sheds the first light on the potentially complex global structure of *Sousa chinensis*. The humpback dolphin appears to adapt behaviourally and ecologically to local conditions, a necessary characteristic of a strictly nearshore species. However, this serves to highlight the potential difficulty in conserving and managing the species on a global scale.

For the present, preservation of the South African population requires the attention of scientists and managers. Many of the suggestions raised in this chapter are highly speculative, emphasising the need for priority research in many parts of the south east African coast. Ideally, the coastline from south of the Tugela Bank through False Bay, would require priority sample collection for genetic analysis. Additionally, samples from north of St. Lucia would be necessary to confirm the suggestion of reproductive isolation between Maputo Bay and South African populations. The elusive nature and low abundance of the humpback dolphin remains the most inhibitive factor to comprehensive research, further complicating the implementation of an effective research programme.

Within the global distribution of *Sousa chinensis*, the South African population appears to exist as a demographically and genetically isolated unit at the edge of the species' Indopacific range. Subdivision within the population, combined with sustained low levels of gene flow may result in fragmentation into allopatric subpopulations. Continual depletion in localised areas would assist in maintaining differentiation as subpopulations shrink toward a focal locality within their range (Wayne *et al.*, 1992; Durham, 1995). Combined with low genetic variability (Chapter 6), fragmentation would serve to reduce the overall genetic fitness and impair the resilience of subpopulations to environmental stochasticity. The KZN Tugela Bank subpopulation represents a stock which is already genetically isolated from adjacent stocks and under constant depletion pressure. Preservation of this population and its unique genetic assemblage requires the implementation of an exacting management and conservation strategy.

CHAPTER 8

FINAL CONCLUSIONS AND RECOMMENDATIONS

8.1 Genetic diversity of south east African inshore dolphins

Four molecular systems tested, including 19 putative allozyme loci, six microsatellite loci, 400 bp of the mtDNA control region and randomly-amplified polymorphic DNA, have demonstrated similarly low genetic variation in bottlenose and humpback dolphins sampled off the south east coast of South Africa. Coupled with their sympatric distribution along the coast, the patterns of genetic variation noted suggest that the diversity and population structure of the two species were shaped by similar evolutionary forces. Consistent with the geological history of the coastline is the proposal that inshore dolphin populations experienced a recent genetic bottleneck, possibly as a result of a decline in sea level and disappearance of the continental shelf at the onset of the last glacial age. Few nucleotide differences in the mtDNA control region of both species suggests that the bottleneck was relatively recent on an evolutionary timescale and that both species have since undergone rapid, but limited radiation.

8.2 Population structure of south east African inshore dolphins

Significant differentiation of mtDNA haplotypes was indicated between East Cape and KZN bottlenose dolphins, but not among putative home ranges within KZN, suggesting that female bottlenose dolphins show regional fidelity to either the East Cape or KZN. Microsatellite allele frequency variance supported this division, revealing similar regional subdivision. That sufficient gene flow occurs to prevent isolation of bottlenose dolphins from the two regions, appears to be the result of the annual 'sardine run' which moves northwards up the east coast.

Humpback dolphins did not display a similar dichotomy in mtDNA genetic variance, displaying significant mtDNA differentiation between localities on the Tugela Bank and those on the rest of the south east coast. Mitochondrial DNA haplotype frequencies further subdivided the Tugela Bank by differentiating samples taken in Richards Bay from those taken elsewhere on the KZN north coast. The significance of these subdivisions implied that humpback dolphins on the Tugela Bank, are strictly philopatric within a small coastal area. Mitochondrial DNA haplotypes and larger coastal habitats support the suggestion by photo-identification data that female humpback dolphins on the eastern and southern Cape coasts may be philopatric within larger ranges than those implied for the Tugela Bank. Contrary to the pattern shown by South African dolphins, Hong Kong humpback dolphins gave no indication of genetic differentiation among mtDNA haplotypes, suggesting that the region sampled forms part of a much larger coastal population.

Microsatellite frequencies supported the mtDNA subdivision of the South African humpback dolphin population into the Tugela Bank and south coast regions. However, high gene flow between Richards Bay and the rest of the KZN north coast indicated that movement does occur among localities within the Tugela Bank region. However, gene flow between the Tugela bank and the south coast is one twentieth of that occurring within the Tugela bank, implying that long range movement between the two regions is severely restricted. While nuclear and mtDNA systems showed similar allele frequencies and identical mtDNA haplotypes in samples from widely separated parts of the south coast, suggesting high levels of gene flow over long distances, this may more likely be a result of sporadic migration by humpback dolphins in response to the 'sardine run'. Finally, significant subdivision of microsatellite allele frequencies in Hong Kong humpback dolphins north and south of Lantau Island indicates that the species displays complex patterns of population structure, determined, possibly, by factors such as habitat suitability, prey abundance, population size and exploitation pressure.

The population structure of inshore dolphins in this study reflects the respective social behaviour of the two species examined. Bottlenose dolphins are gregarious, occurring in large schools. These are characteristics which would facilitate the regular long range movements required to produce the structure evident from the data. Furthermore, greater mobility afforded by larger schools means that bottlenose dolphin distribution is not dependent upon habitat suitability. By contrast, the elusive behaviour of humpback dolphins and their occurrence in small schools severely restricts long range movement. The species' preference for shallow water further limits its distribution along a coast characterised by a narrow inshore area. Consequently, a patchy distribution and restricted movement has produced a significantly subdivided population.

8.3 Implications of genetic results for the conservation of inshore dolphins

If the evolutionary history of South African coastal dolphins, as proposed in this study, is correctly assumed, then it is likely that bottlenose and humpback dolphins along the entire south east coast, are characterised by similarly low genetic variability. In the absence of an infusion of genetic variation, continued exploitation of inshore dolphins off KZN may further reduce genetic diversity to a point that may compromise the resilience of both species to future stochastic occurrences.

Significant genetic differentiation in the bottlenose dolphin population implies that regular movement of individuals between KZN and the East Cape is restricted. Thus, unless physical recruitment of individuals into KZN occurs during the sardine run, the influx of dolphins from the East Cape will not have a meliorating effect on mortality. More important is the effect of female philopatry on depletion of the KZN population. The significant degree of mtDNA differentiation suggests that the movement of female bottlenose dolphins is somewhat restricted. This has important implications for the KZN population; nearly 70 % of the bottlenose dolphins captured in shark nets are mature females and calves (Cockcroft and Ross, 1991). If females are philopatric, not only are they more likely to be captured in shark nets, but replenishment of females from adjacent areas is unlikely. In the face of continued exploitation, the KZN stock will suffer demographic imbalance and the reproductive rate will ultimately decrease. In the absence of regular recruitment, this would lead to a systematic decline in the population.

The same situation would apply to humpback dolphins. However, while movement of female bottlenose dolphins within KZN may offset local depletion, strict fidelity of female humpback dolphins to small areas may result in accelerated depletion in some localities. Furthermore, restricted long range movement of male humpback dolphins implies that replenishment from distant localities would not occur. Subject to confirmation by analysis of dolphins from unsampled areas, it appears from this study and that of Durham (1995), that the entire KZN humpback dolphin subpopulation may be concentrated on the Tugela Bank. A lack of immigration from areas north and south of this region, will not only prevent an infusion of genetic variability, but without physical recruitment, the natural replenishment rate would be unable to offset the high capture rate in areas such as Richards Bay. In light of the significant differentiation of the humpback dolphin population, the threat of local extinction on the Tugela Bank should be seriously considered in the face of continued incidental capture. Given the degree of philopatry and the inshore tendency of the species, extinction of the KZN subpopulation will doubtless isolate the eastern and southern Cape subpopulation from humpback dolphin populations elsewhere on the African east coast. This would, ultimately, increase the likelihood of extinction of the species from the South African coast.

8.4 Recognition and management of inshore dolphin stocks

Recently, the definition of the evolutionarily significant unit (ESU), as a means of defining taxa for conservation, elicited much discussion (Ryder, 1986; Dizon *et al.*, 1992; Moritz, 1994a). Moritz (1994a) concluded that population units should be defined according to their degree of evolutionary divergence. He proposed that the term ESU should be designated to populations which are differentiated to the extent of reciprocal monophyly. Less divergent populations, which demonstrate gene flow, but differ significantly in allele frequencies at nuclear or mtDNA loci are defined as management units (MU; op cit).

Delegates at a workshop on the analysis of genetic data to address problems of stock identity related to management of cetacean populations recommended that MUs be based on subdivisions indicated by the mtDNA, regardless of the degree of male-mediated gene flow. It was noted that females are the source of subsequent generations, thus by managing a population in terms of its maternal differentiation, the true structure of the population will always be revealed (Dizon *et al.*, 1997). Maternal genetic differentiation in humpback whales was used to define major stocks which appeared to result from fidelity to migration routes (Baker *et al.*, 1993). From these data, humpback whales were determined to represent a single ESU, comprised of several maternally-differentiated MUs. By comparison, significant divergence in allele frequencies in both nuclear and mtDNA loci are consistent with placing bottlenose dolphins from KZN and the East Cape into separate management units. Similarly, humpback dolphins in the KZN Tugela Bank region can be distinguished as a management unit separate from those occurring off the eastern and southern Cape coasts.

The risk-averse strategy for defining stocks as formulated by the workshop, recommends that, unless panmixia can confidently be assumed, populations should be considered separate in the face of any ecological, morphological, genetic or life history evidence suggesting distinctiveness. A simulation of a management situation applicable to marine mammals was presented to illustrate the consequence of failing to distinguish populations under a risk-averse strategy (Taylor, 1994). Two subpopulations of equal size were considered, with incidental take occurring in one subpopulation. This approximates the situation in this study, where incidental take occurs only in KZN. The goal was to maintain the population as a whole at or above 50 % of its carrying capacity and this was assessed under three models of dispersal, including fixed density in both subpopulations, density-dependence in the emigrant subpopulation and density-dependence in both emigrant and immigrant subpopulations. In all three cases, the exploited subpopulation faced extinction when dispersal was less than 2 %, and the whole population failed to reach 50 % of its carrying capacity. The simulation illustrated that by ignoring population structure and managing differentiated subpopulations as a single population, depletion can occur as a result of concentrated exploitation. This comparison is valid for South African dolphins, where for both species, the estimated dispersal rate between KZN and East Cape stocks is less than 2 %.

It is proposed that a risk-averse management strategy be adopted for both bottlenose and humpback dolphins. As a null hypothesis, dolphins from the KZN and East Cape are assumed to comprise a single population. Based on evidence from this study, the null hypothesis may be rejected. However, if the data have over-estimated population subdivision and the null hypothesis has been incorrectly rejected, it would represent a type I error (rejecting a null hypothesis when it is true). This would err towards the conservative and would ensure conservation attention to the exploited stocks. Thus the regional segregation evident from the genetics of both bottlenose and humpback dolphin populations should be used to define management units for the purpose of conserving the species on the south east African coast and more particularly, on the KZN coast. Humpback dolphins from the Tugela Bank region should be managed separately from humpback dolphins elsewhere on the coast. Similarly, bottlenose dolphins off KZN should be managed as a separate stock from bottlenose dolphins in the eastern and southern Cape. This would imply that, contrary to the proposal of Peddemors (1995), the annual migration of bottlenose dolphins into KZN cannot be relied upon to offset the suspected depletion occurring in the netted region of KZN.

8.5 Recommendations

8.5.1 Future genetic studies

The investigations conducted in this study should be extended to bottlenose dolphin schools north of the netted region in KZN in order to determine whether the KZN coast comprises a single subpopulation or whether it can be further subdivided. The presence or absence of additional structure within KZN would have crucial implications on the management of bottlenose dolphins in that region. Furthermore, determination of the degree of residence demonstrated by bottlenose dolphins is required, to determine with confidence, the impact of shark net mortality on putative resident schools in KZN. This may be achieved by co-operative genetic and photo-identification studies along the length of the south east African coast. Data from this study indicate that an investigation of hypervariable minisatellite DNA or the resolution of several species-specific microsatellite loci may provide the most informative results. However, unless numerous clear markers are resolved, it may not be possible to distinguish, genetically, animals from closely adjacent localities.

Additional information is required regarding the structure of the eastern and southern Cape humpback dolphin population. However, the elusive and boat-shy nature of the species makes biopsy sampling of free-ranging humpback dolphins difficult. The degree of reproductive isolation of humpback dolphins along the South African coast from those off Mozambique requires urgent assessment. The slaughter and incidental capture of humpback dolphins in Maputo Bay has been reported (Guissamulo, 1993; Cockcroft and Krohn, 1994) and the collection of samples for genetic study must become a priority for researchers in that region.

8.5.2 Conservation priorities

Although conservation and management of bottlenose dolphins on the whole south east coast is required to ensure the sustainability of the species in the south western Indian Ocean, protection of the KZN stock should be a priority in order to facilitate recovery of the population from prolonged exploitation. In light of the indication by data in this study that female bottlenose dolphins are regionally philopatric, the high rate of mortality of females and calves in shark nets requires serious consideration.

Low population number makes the immediate conservation of humpback dolphins in KZN a priority. Furthermore, the heavy exploitation of humpback dolphins in shark nets off Richards Bay requires urgent re-evaluation in view of the strict fidelity shown by the species to that locality.

Conservation of the coastal habitat is a prerequisite to ensuring long term persistence of inshore dolphins. However, simply preserving habitat is insufficient where populations are small, fragmented and demographically compromised (Mace and Lande, 1991). Given the recorded catch statistics to date, it has been speculated that between 1000 and 2000 bottlenose dolphins have been caught since the shark nets were first installed in Durban (Cockcroft, personal communication). This at least equals the number of calves born during that period, assuming an annual growth rate of 3-5% (Reilly and Barlow, 1984), suggesting that over four and a half decades of net captures have almost certainly resulted in depletion of both species in KZN. It is clear that the drafting and implementation of conservation and management policies concerning inshore dolphins in South Africa would require the cessation of unchecked exploitation in KZN. This would entail, at least, the removal of shark nets from coastal regions supporting the greatest density of inshore dolphins and certainly from regions identified as nursery areas for both dolphin species.

8.5.3 Concluding remarks

Coastal dolphins are exposed to anthropogenic activities throughout the Indo-Pacific region (Cooke, 1991; Obura *et al.*, 1996). Since the region comprises mainly developing countries, habitat destruction and exploitation, both incidental and deliberate are contributing rapidly to declines in coastal populations (Cooke, op cit; Lal Mohan, 1985, 1994). Conservationists and policy-makers in South Africa are obliged to enforce protective management of coastal fauna, not only because the region represents the edge of the Indo-Pacific basin, but because South Africa is one of a handful of countries in the Indo-Pacific region with the resources to do so. Although the data from this study require validation from additional data, the trends and their implications noted are sufficient to indicate that failure to implement concerted management strategies aimed at reducing deliberate exploitation may result in severe depletion and ultimately, local extinction in regions of the South African coast.

Intensive research has been directed at KZN inshore dolphins in the past decade (Cockcroft, 1990b, 1992; Cockcroft *et al.*, 1989b, 1990b, 1991b, 1992; Cockcroft and Krohn, 1994; Cockcroft and Ross, 1989, 1990, 1991; Durham, 1995; Goodwin *et al.*, 1996; Peddemors, 1995; Peddemors *et al.*, 1991; Ross *et al.*, 1987, 1989), the data of which have implied that bottlenose and humpback dolphins in that region are subject to population depletion as a combined result of fragmentation and degradation of suitable habitat, continued incidental capture and a high rate of natural mortality, all of which is further aggravated by the accumulation of toxic levels of pollutant contamination. Nonetheless, this appeared to be insufficient motivation for concerted action against factors contributing towards depletion. The genetic data from this study have contributed to defining, by implication, the movement and interaction of bottlenose and humpback dolphins from KZN with those from adjacent regions. There is now sufficient information on both species to support active conservation and protection from further exploitation and habitat loss.

Failure to conserve KZN inshore dolphin stocks will not only assist in isolating remaining South African populations, contributing further to the fragmentation of coastal bottlenose and humpback dolphins stocks in the western Indian Ocean, but it will contribute substantially to the conservation apathy which is prevalent in many countries bordering that ocean. The preservation of marine ecosystems and conservation of sustainable resources are practices which still require implementation in the developing world. Having set several precedents in the conservation of terrestrial fauna, South Africa is now obliged to extend conservation efforts to coastal marine fauna and their habitat.

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APPENDIX I

Consensus sequence for the first 400 bases of the bottlenose dolphin mtDNA control region. Nucleotide position is recorded from 5' to 3' on the mtDNA heavy strand corresponding to the region from similarity block E to tRNA^{PRO} in Southern *et al.* (1988).

```
                                                               60
ACCCCCACGA GATATGGGCC CGGTGCGAGA AGGGGGATCC CTGCCGAGCG GGTTGCTGGT

                                                              120
TTCACGCGGC ATGGTGATTA AGCTCGTGAT CTAATGGAGC GACCATAGGA TACAATGGAG

                                                              180
ATGAAATTGA -AGGGGATAT ATAATATGTA AGAGTATGCA TATTATGTAA TATGTAAAAT

                                                              240
TAATGATAAT TAATACGAGC TTTAACTTAT CGTATGGAAA ATAAATGAAT GCACAATAAT

                                                              300
ACATAGCATG TATATATGAA TATTTACAAG GAGAGACTAA ATATTAGCAT GTACATGTGT

                                                              360
ATGTGTATGT AATGTGTACA GTAAAATGTT TTTAAAACAA ATTACTTTTA ATACTGACGT

                                        400
AGTACTGTGA TGTTGTGGTA ATTGTACAAT AAGCTTTTTC
```

APPENDIX II

Consensus sequence for the first 400 bases of the humpback dolphin mtDNA control region. Nucleotide position is recorded from 5' to 3' on the mtDNA heavy strand corresponding to the region from similarity block E to tRNA^{PRO} in Southern *et al.* (1988).

```
                                                              60
ACCCCCACGA GATATGGGCC CGGTGCGAGA AGGGGGATCC CTGCCGAGCG GGTTGCTGGT

                                                             120
TTCACGCGGC ATGGTGATTA AGCTCGTGAT CTAATGGAGT GGCCATAGGA TATGATGGAA

                                                             180
ATAAAATTGA TAGGGGATAT ATAATATGTA AGAGTATGCA TATTATGTAA TATGTAAAAT

                                                             240
TAATGATAAT TAATACGGGC TTTAACTTAT CGTATGGAAA ATAAATGAAT GCACAATAAT

                                                             300
ACATAGCATG TATGTATGAA TATCTACAAG GAGAGACTAA ATATTAGCAT GCATGTGTGT

                                                             360
CTGTGTATGT GATGTGTACA GTAAAATGTT TTTAAAACAA ATCATTTTTA TAGCTGGCGT

                                        400
AGTACTGTGA TGTGTGATGA TTGTACAATA AGCTTTTTC
```