Genetic structure of sperm whale populations assessed by mitochondrial DNA sequence variation

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by Mary Catherine Dillon

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DEDICATION

This thesis is dedicated to my grandfather, C.R. Williams, and a dear family friend, Laura McGurk, for the influence they had on the early years of my life.

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ABSTRACT

Working from DNA extracted from skin naturally sloughed by free-living sperm whales (*Physeter macrocephalus*), I amplified and sequenced the mitochondrial control region to investigate (1) the evolution of this molecule; (2) the social organization of groups of female and immature sperm whales; and (3) the population structure of sperm whales within and among three major ocean basins.

First, the DNA sequence of the control region of sperm whales was compared to that of other cetaceans. The control region was reported previously to contain sequence blocks that were highly conserved among cetaceans, and assumed to have critical functions. Comparison of the sperm whale sequence, however, demonstrated that many of the sequence blocks that had been observed to be well conserved, were very different in sperm whales, calling into question the importance of these sequence blocks in at least some cetaceans.

Second, organization of three social groups of female and immature sperm whales was examined using sequences from the most variable part of the control region. Analysis of resulting haplotypes revealed that group members were related, and that the groups had a matrilineal structure. A comparison of mitochondrial data to nuclear data from the same individuals indicated that some group members may be paternally related.

Finally, examination of mitochondrial haplotypes in the Pacific (N=140), Atlantic (N=23) and the Indian (N=19) oceans also showed the relatedness of members of social groups. However, little genetic structure above the level of groups was evident. Almost none of the total genetic variation observed was due to differences between 3 widely-separated areas in the S. Pacific, nor to differences in the three major ocean basins. This study also revealed a lack of genetic variation in sperm whales world wide.

LIST OF ABBREVIATIONS

A	adenine
AMOVA	analysis of molecular variance
bp	nucleotide base pairs
C	cytosine
°C	degrees celsius
Ci	Curie
CSB	conserved sequence block
dATP	deoxyadenosine-5'-triphosphate
ddH_2O	deionised, distilled water
df	degrees of freedom
D-loop	displacement loop
DMF	dimethyl formamide
dNTP	deoxyribonucleoside triphosphate
ddNTP	dideoxyribonucleoside triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fig.	figure
G	guanine
h	hour(s)
HCl	hydrochloric acid
IPTG	isopropylthio-β-D-galactoside
IWC	International Whaling Commission
Kbp	kilobase pairs

KCl	potassium chloride
M	molar
mA	milliampere
mg	milligram
$MgCl_2$	magnesium chloride
min	minute(s)
mL	millilitre
mM	millimolar
mt	mitochondrial
mtDNA	mitochondrial DNA
N	sample size
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
p	probability
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	phenylalanine
Pro	proline
RE	restriction endonuclease
RF	replicative form (of M13 DNA)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rotations per minute
s	second(s)
SDS	sodium dodecyl sulfate

T	thymine
Thr	threonine
tRNA	transfer RNA
TE	tris-EDTA
TAE	tris-acetic acid-EDTA
TBE	tris-boric acid-EDTA
U	units
μg	microgram
μL	microliter
UV	ultra violet
V	volts
v/v	volume per volume
W	watts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
yr	year(s)

ACKNOWLEDGEMENTS

I feel very fortunate to have had the opportunity to do thesis work that combined the fields of molecular genetics and sperm whale biology. I thank Drs. Jonathan Wright and Hal Whitehead for collaborating to allow this project to happen. My sincere thanks go to Jonathan for overseeing this project, giving guidance and encouragement, and for his generosity in terms of personal financial support, conference travel and lab expenditures. I thank Hal for giving me the opportunity to go to sea to study whales. Swimming with sperm whales was definitely a highlight of my thesis. I also thank Hal for his interest in my work, but also his patience with the often slow progress of this research. Hal's assistance with analysis and statistics was invaluable.

Many committee members had input into various stages of this thesis and I thank Drs. Paul Bentzen, Marty Ball, and Ian McLaren. Drs. Michael O'Connell and Don Stewart provided feedback on manuscripts and useful discussions of my work. Dr. Tom MacRae was an honourary committee member, who provided information and advice on all things academic, and who was always willing to provide a diversion from work by talking hockey. Nice guy for a Habs fan!

The availability of samples from around the world made this thesis much more interesting. I thank everyone who had a part in collecting skin for this work. In particular, I thank those who organized field work and sailed their own boats: Hal

Whitehead in the South Pacific, Benjamin Kahn in the Indian Ocean, and Jonathan Gordon and Michel André who provided Atlantic samples from the Azores and the Canary Islands, respectively. Also, I thank the members of *Balaena* who put extra effort into sample collection, especially Simon Childerhouse, Nathalie Jaquet and Kenny Richard.

Kenny Richard did much of the initial research into collecting skin samples and extracting DNA. His work paved the way for mine, and I acknowledge his contribution. Kenny also provided me with his microsatellite data so I could include a comprehensive study of sperm whale social organization in this thesis. Susan Waters and Susan Dufault catalogued and analysed the fluke identification photographs, which forms the basis for so much sperm whale work, including mine.

I thank my "Alberta connection", Jens Franck and Pat O'Reilly for support, friendship and assistance with all things technical. Jens was tremendously helpful in getting me started in the lab, showing me around and answering all my questions. I sincerely appreciate his interest, support and encouragement. Pat always seemed to be helping someone in the lab, and I was on the receiving end of my share of his assistance. I also thank him for all the discussion about my work and useful troubleshooting ideas.

I really enjoyed working in the lab, and I am fortunate to have worked with so many great people. Thanks, also to

some more distant lab mates at CBC radio, especially those who put together "Morningside". CBC radio was my constant companion in the lab, and I don't know how I would have passed all those hours without it.

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PUBLICATIONS

Some of the research in chapter 3 also appeared in:

Dillon, M.C. and Wright, J.M. 1993b. Nucleotide sequence of the D-loop region of the sperm whale (*Physeter macrocephalus*) mitochondrial genome. **Molecular Biology and Evolution 10:** 296-305.

Some of the research in chapter 4 also appeared in:

Richard, K.R., Dillon, M.C., Whitehead, H. and Wright, J.M. 1996. Patterns of kinship in groups of free-living sperm whales (*Physeter macrocephalus)* revealed by multiple molecular genetic analyses. **Proceedings of the National Academy of Sciences, U.S.A. 93:** 8792-8795.

(This research represents a comparison of microsatellite and mtDNA data. Kenny Richard carried out the microsatellite assays and analysis. I performed all the mtDNA work. Modelling was conducted by Hal Whitehead. In this chapter I used "I" to identify the mtDNA work done by me, and "we" to reflect the collaborative effort put into the synthesis of the 2 datasets).

Some of the research in chapter 5 has been submitted for publication:

Dillon, M.C., Whitehead, H., and Wright, J.M.
Genetic structure of populations of female and immature sperm whales (*Physeter macrocephalus*) assessed by mitochondrial DNA sequence variation over a range of social and geographic scales. Submitted to **Molecular Ecology.**

CHAPTER 1: A review of cetacean genetic studies and potential applications for population studies of sperm whales (*Physeter macrocephalus*)

Introduction

The complex social systems and interesting life histories of many cetacean species have made them obvious targets for genetic analyses. Genetics studies are being used to investigate many aspects of cetacean biology, including the degree of mixing between populations (to determine a genetic basis for stocks), the amount of genetic variation within populations (for management and conservation), and the genetic basis of social organization (to assess the role of kin selection). However, collecting samples from some whales and dolphins is difficult. Among the most challenging of cetacean species to study is the sperm whale (*Physeter macrocephalus*). These whales are found in all oceans of the world, but generally only in deep waters. Therefore, one usually needs to be equipped to go to sea for weeks at a time in order to observe these animals. This is in contrast to some coastal species that can easily be observed from cliffs or small boats. In spite of the effort required to study these animals, they are an appealing and important species, with a complex social structure and unique biological features. In this chapter, I review briefly the biology of the sperm whale, how molecular population genetics studies have increased our

knowledge of cetacean biology, and discuss potential applications in the sperm whale, especially in terms of assessing stock boundaries.

The biology of the sperm whale

The sperm whale is an animal of extremes: it is the largest odontocete, it has a huge head that may be a full one third of a mature male, with the largest brain of any animal that has ever lived. Differences in the size of male and female sperm whales are greater than in any other living cetaceans, with mature males reaching 1.5 times the length of mature females, and up to 3 times the mass. The sexes also have greatly different distributions, with females and immature whales having a much more restricted range than large males, whose range is only exceeded among mammals by the orca (*Orcinus orca*). Sperm whales are among the deepest diving of all cetaceans (rivalled by the beaked whales), and with the other Physteridae, share a karyotype that is unique amongst cetaceans (Arnason and Benirschke, 1973). They are the only cetacean in which the atlas (first cervical vertebrae) remains free, they are unusual amongst cetaceans in that the height of the scapula exceeds its width, and their nasal passages are more asymmetrical than in the other odontocetes (Rice, 1989). In fact, the sperm whale is so unusual in many respects that Dale Rice, a whale biologist, has been reported to have said that if it didn't already exist, one might think it impossible (Bonner, 1989). It is surprising, then, that despite these

unique attributes, the sperm whale has been referred to as the "generic" whale (Hoyt, 1984), the stereotypical animal one imagines when thinking of whales. This could be due to the sperm whale's long history with man in the whaling industry and/or the infamy of the sperm whale due to Herman Melville's classic "Moby Dick". The natural history of this species, with emphasis on the social structure, patterns of movement of males and females, and what is known about stock divisions, is presented here.

Social structure

One of the most interesting features of sperm whales is their social structure. In tropical and subtropical waters, "mixed" groups of sperm whales are found. These schools consist of females of all ages and stages of reproduction and their young of both sexes; these groups are accompanied by mature males during the mating season. There is evidence that these groups consist of related females, who may stay together for long periods of time. Oshumi (1971) reported that females marked in the same schools have been found together as much as 10 years later. Using passive techniques, Gordon (1987), Whitehead and Waters (1990) and Whitehead *et al.* (1991) have also observed mixed groups over periods of years, and have reported that at least some of the group members have remained together. The size of these presumably matricentric groups has usually been considered to be about 20 individuals; however, Whitehead *et al.* (1991), using sighting data from the

Galápagos Islands that spanned 4 years, revised the ideas of the composition of these female groups. They suggested that the individuals that stay together over time are really "units", with membership of about 12. Units can come together to form groups of about 23 individuals; these associations may last for only a few days. The authors speculate that the function of the units relates to communal care of calves, while the groups that form may have to do with efficient foraging. In support of the idea of communal care of calves, Arnbom and Whitehead (1989) observed calves being escorted by more than one individual, and adult females were seen with more than one calf on different occasions. Whitehead (1996) has shown that members of a group in which there is a calf change their diving patterns in order to minimize the time a calf is left alone at the surface when its mother dives. Gordon (1987) also reports that calves are often seen being escorted by individuals other than their presumed mothers, and suggests that calves may suckle from females that are not their mothers. It has long been speculated that groups are made up of related individuals, but testing of this hypothesis required that genetic studies be applied to sperm whales. Recently, Richard (1995) investigated relatedness in groups of sperm whales using microsatellite loci; he found that whales in the same social group shared more alleles than whales in different groups, and that the group structure was consistent with one or more matriline(s).

In comparison, associations of males are quite different. Males, while still immature, leave the mixed groups and join "bachelor schools". Best (1979) has described three types of male schools: groups of small bachelors (with size range about 10-12m), medium bachelors (12-13.7m) and large bachelors over 13.7m. It seems that these bachelor schools are more homogeneous for size than for age (Best, 1979); groups of small males which may have a size difference of only 1.8 m can have an age range of 20 years. Bachelor schools have much looser organization and membership than the mixed schools, such that as males get older, they seem to be found in smaller and smaller groups, with ranges into higher and higher latitudes. The largest males are usually found singly, only joining mixed schools for breeding. Males are thought to achieve sexual maturity around age 18-19 years (Rice, 1989) but are not socially mature, taking an active part in breeding, until age 25-27 years (Best, 1979). The traditionally-held view was that these socially mature males controlled a "harem" of females and fought with other males for exclusive breeding rights in a mixed group. More recent work, however, has shown that these breeding bulls employ a searching strategy, moving between groups of females (Whitehead, 1993). Whitehead (1990a) has shown that it will be more profitable for males to employ a searching rather than a harem-holding strategy if the mean time to encounter a new mixed group is shorter than the oestrus period of the females.

Movements of male and female sperm whales

It is clear that the movement of males is much more extensive than that of females. Mixed groups are generally confined to tropical and subtropical waters, about 40°S to 40°N (*e.g.* Best, 1979; Rice, 1989). Males (as a whole) on the other hand, have been reported to range from 70°S to 78°N (reviewed by Rice, 1989). While the distribution of the species spans deep waters in all oceans of the world, the major continental land masses are thought to represent a barrier to movement of mixed groups. Movement of mixed groups may be possible between the Atlantic and Indian Oceans, and the Indian and Pacific, but it is almost certain that movement would be restricted between the Atlantic and Pacific; if any animals rounded Cape Horn, it would be only the largest males. Ivashin (1981) reviewed information on movement between eastern and western South America. It seems that these whales have separate breeding grounds, but the possibility of mixing between males of different stock divisions in Antarctic waters is not ruled out. Although movement between oceans is almost certainly limited, there is much evidence of extensive movement within ocean basins. Rice (1989) reviewed mark and recovery data and concluded that there is extensive seasonal movement in both hemispheres, which opens up the possibility of genetic exchange within ocean basins. Despite this avenue for exchange, there is some evidence of morphological, immunogenetic and enzyme differences in local populations of

sperm whales within oceans (reviewed below).

Stock assessment in sperm whales

The definition of what constitutes a "stock" is of obvious importance for management of species which are whaled, assessment of the effects of past whaling, and decisions about future whaling.

Definition of a stock

The effective biological unit in whales is not the species but "the breeding population of local stock" (Allen *et al.*, 1971). Two concepts of stocks are in use. The first is that of a biological stock, defined by the International Whaling Commission (IWC) in 1976 as "a relatively homogeneous and self-contained population whose losses by emigration and accessions by immigration, if any, are negligible in relation to rates of growth and mortality" (Donovan, 1991). Unfortunately, for most cetacean populations, not enough data are available to make an accurate assessment of biological stocks. More commonly, the IWC uses the idea of management stocks, which are defined as populations that can be 'successfully' managed (quotation marks mine). This, however, is problematic, as virtually no cetacean populations which have been subject to substantial exploitation have been successfully managed. For example, Tillman (1977) asserts that managing Southern Hemisphere fin whales (*Balaenoptera physalus*) as one population masked the fact that one stock was still at a level that could be exploited. However, it is the

reverse problem that is more serious; namely, overharvesting a particular stock under the assumption that the overall population (or aggregation of populations) is healthy.

Virtually all of the knowledge about sperm whales before the last decade came from whaling operations (an exception being mass strandings of presumed entire groups). Methods used to define the boundaries between stocks included catch per unit effort statistics (*e.g.* Gambell, 1976), length specific models (Cooke, 1986), morphological characteristics (*e.g.* morphology of tail fluke notches, Veinger, 1980), blood group analysis (Cushing *et al.*, 1963; Fujino, 1963), and allozyme variation (*e.g.* Wada 1980).

North Pacific sperm whales

Sperm whale stocks have been best studied in the North Pacific, presumably due to Russian and Japanese interest in whaling, and the availability of samples in this area. However, most of the data in this area are from large males (who, because of their size, were preferred over females by whalers).

Initially, the north Pacific was treated as a single stock. However, blood typing work, using the Ju locus, suggested that Japanese and Aleutian whales represent separate stocks (Fujino, 1963). Other work based on sperm whale distribution and differences in seasonal size composition suggested three stocks, roughly localized in waters off Japan, the Aleutian Islands, and North America (reviewed by Best,

1974; Bannister and Mitchell, 1980). Additionally, the use of fluke notch morphology (Veinger, 1980) suggested three separate stocks, where sperm whales in central, eastern and western North Pacific had different distributions of fluke notch types. However, this study was only suggestive of differences based on fluke morphology, and not convincing. The three different notch morphologies were not defined, sample sizes were not given, and no statistical tests were performed.

It is demonstrative of the difficulty in assessing and managing sperm whale stocks that although more data are available for sperm whales in the North Pacific than anywhere else in the world, no agreement has been reached about North Pacific stocks. It is not surprising, then, that sperm whale stock divisions are not clear in other parts of the world, where much less research has been directed.

Southern Hemisphere sperm whales

The first discussion by the IWC of Southern Hemisphere stock identification of sperm whales was in 1963. The Committee noted the variety of techniques available for assessing stock boundaries (tagging, blood typing, time of migration and catch compositions, sightings, parasites, morphological features), but didn't seem to use any of these; instead, they accepted the stock divisions set out for baleen whales and said that these would be altered as sperm whale data became available (Donovan, 1991). In one such stock

division alone, Dufault and Whitehead (1995) found evidence of three separate geographic stocks, in that whales from these widely-separated areas did not appear to mix. Although little work on assessments of Southern Hemisphere sperm whales has been done since the preliminary work in the 1960's and 1970's, Ivashin (1981) conducted a mark-recapture study, noting that most recaptures were in the marking area or adjacent waters, giving support to the idea of local populations of sperm whales. The same author also states, however, that both males and females are capable of extensive migrations. Due to the low number of mark recoveries, it is difficult to determine the amount of mixing between local groups, and the issue remains unresolved.

North Atlantic sperm whales

The entire North Atlantic is considered a single management unit, based on evidence that includes a whale tagged off Nova Scotia being killed off Spain, and length data analysis. However, the Scientific Committee of the IWC has admitted that there is a paucity of knowledge of North Atlantic sperm whales (Donovan, 1991).

Equatorial Pacific sperm whales

The waters of the equatorial Pacific and the Galápagos Islands were traditional sperm whaling grounds (Townsend, 1935; Bannister and Mitchell, 1980). It seems surprising, then, that relatively little work was done on assessing stocks in this area (given the amount of work that was going on for

North Pacific sperm whales). Only a few studies in this area have been carried out, and little work on stock assessment has taken place. In 1958 and 1959, whale surveys were conducted off the coast of Chile and Ecuador (Clarke, 1962), and species and numbers of whales encountered were recorded. Rice (1977) also undertook a survey of the eastern equatorial Pacific, but again, it was essentially a descriptive study documenting the number of whales encountered, estimating their size and school composition and so on. The only mention of stocks is a speculative suggestion that equatorial populations are composed of Northern Hemisphere animals during the boreal winter and of Southern Hemisphere animals during the austral winter. Laake and Hammond (1984) and Wade and Gerrodette (1993) have estimated sperm whale group size and density in the eastern tropical Pacific using sighting data, but made no mention of stock divisions.

There are a few important points to make. First, it is clear that, at present, there is not enough information to make accurate assessments of sperm whale stocks, even though management and conservation decisions should be based on such information. Second, the traditionally-important equatorial Pacific and Galápagos grounds represent a group of sperm whales that are very interesting because they may contain both Northern and Southern Hemisphere whales; clearly, work on stock assessment in this region is important. Finally, in the absence of sperm whaling (which was banned by the IWC in

1985), the usual methods of stock assessment (mark and recapture, catch distributions, morphology *etc.*), which are almost entirely based on killing whales, will not be appropriate.

Studies of sperm whales that do not depend on catches

The realization that much could be learned about sperm whales without killing them has allowed us to increase our knowledge of sperm whales in this post-whaling period. Whitehead and Gordon (1986) describe methods for non-invasively studying sperm whales. These methods include identifying individuals using natural tail fluke markings (Arnbom, 1987; Whitehead, 1990b), estimating lengths from photographs (Gordon, 1990), analyzing diet using defecated squid beaks (Smith, 1992; Smith and Whitehead, 1993) and investigating the behaviour and number of sperm whales present using acoustic studies (Gordon, 1987; Whitehead and Weilgart, 1990; Weilgart and Whitehead, 1993). These non-invasive methods have also been applied to assessing stock divisions, so that the absence of whaling has not prevented further investigation into this area. In the Galápagos area, observations suggest that the main calving period is around June (Whitehead *et al.*, 1989), which is consistent with those whales being on a Northern Hemisphere schedule. This contrasts with the situation off the coast of South America, where very small calves were observed in January/February off Ecuador (personal observations) and where Clarke *et al.* (1980)

have suggested a Southern Hemisphere schedule based on whaling records from Paita, Peru. Although the whales off the Galápagos and mainland Ecuador/Peru are not widely separated geographically, they may be isolated from each other reproductively, in that they appear to be six months out of phase with each other. Thus, there is a possibility of stock divisions based on reproductive schedule.

Individual identifications using photographs also shed light on stock divisions. While it is known that some females move to different regions within an ocean basin (*e.g.* whales identified in the Galápagos in one year being reidentified off Peru later (marking and recovery data, Ivashin, 1978; fluke photograph data, Dufault and Whitehead, 1993), longitudinal studies by Whitehead and colleagues in the Galápagos, and by Gordon (1987) off Sri Lanka have identified the same whales in the same geographical area after one year or more, suggesting site fidelity. If some females make only limited movements within an ocean basin, the possibility of finding discrete stocks in that ocean is increased. In fact, evidence of separate stocks in the South Pacific is being accumulated. Whitehead *et al.* (1992) used photographs taken over a 4 year period in the Galápagos Islands and estimated population parameters using a modified mark and recapture model. They concluded that mixed groups observed around the Galápagos are part of a larger population of a few thousand animals, and that within an ocean basin, groups of females and immatures

may form small stocks. Further evidence of stock division comes from a study by Dufault and Whitehead (1993), who found that their null hypothesis of complete mixing between whales identified off the Galápagos and Ecuador over a two year period was not supported based on fluke identifications; only 5% of the whales identified in the Galápagos had been reidentified in Ecuador, and no Ecuador whales have been later sighted in the Galápagos (although there are fewer data available to assess movement in this direction). In addition, there have been no instances of a whale identified or marked in the Galápagos or Ecuador/Peru being re-identified in the E. south Pacific south of 10° S, nor in the W. south Pacific in photographic identification studies (Dufault and Whitehead, 1995), or in mark-recapture studies (Ivashin, 1978). This lends further support to the possibility of stock separation in the Southeast Pacific.

Whitehead *et al.* (1992) suggested the possibility of small stocks around the Galápagos, but admitted that the degree of separation of these stocks and their geographical ranges are not known at present, and that genetic analysis may be important in answering questions about these stocks. Indeed, genetic analyses are proving to be extremely useful in the study of cetaceans. These studies have been facilitated by the ability to collect samples non-invasively (*i.e.* collecting sloughed skin, Whitehead *et al.*, 1990; Amos *et al.*, 1992) and non-consumptively (using biopsy darts, *e.g.*

Palsbøll *et al.*, 1991; Brown *et al.*, 1991; Weinrich *et al.*, 1991), and by developments in molecular biology.

DNA techniques used to study population biology

1. Nuclear variation

Minisatellites

Much interest was generated when it was reported that hypervariable minisatellite regions in human DNA could be used to identify individuals unambiguously, using the method of DNA fingerprinting (Jeffreys *et al.*, 1985a, b). Minisatellites are short stretches of DNA which are tandemly repeated 10's to 100's of times, and are spread throughout the genome. Hypervariability is detectable as variation in the number of repeats, believed to be generated by loss or gain of repeat units during recombination (Jeffreys *et al.*, 1985a, 1988). The DNA fingerprint is generated by probing blots of size-fractionated, restriction-digested genomic DNA with a sequence that hybridizes to the "core" sequence that many minisatellites share.

DNA fingerprinting has been applied to individual identifications for forensic and immigration cases, to medical problems (such as studies of tumours, analysis of inherited diseases, bone marrow transplants; Wong *et al.*, 1987; Burke, 1989), but the main application of DNA fingerprinting studies to date has been paternity analysis. Using this method, the closest relatives of an individual can be positively identified, as opposed to other approaches that can only

exclude individuals from being closely related. The implications for ecological studies go beyond the mere identification of a father; determination of pedigrees is important for understanding and predicting dispersal patterns and aspects of behaviour.

One complication with multilocus fingerprints is that, since multilocus probes examine many loci at the same time, one cannot link a specific band to a specific locus, and relationships more distant than parent-offspring or full sibs can be obscured (Tautz, 1990).

Attempts to overcome the limitations of multilocus probing have involved the use of single locus probes (Wong *et al.*, 1986, 1987), such that alleles can be assigned to specific loci. It is thought that these may be valuable markers for mapping human genetic diseases (Nakamura *et al.*, 1987). However, the use of this technique has been slowed by difficulties in isolating probes, and has not been widely applied to non-human studies.

Microsatellites

Some of the difficulties associated with minisatellites may be overcome with the use of microsatellites. These sequences occur in high copy number throughout the genome and consist of di-, tri-, or tetra-nucleotide motifs which are tandemly repeated. Since these "simple sequences" were recognized as useful genetic markers (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989), they have been

characterized in a wide variety of species. Most microsatellite work takes advantage of the fact that these simple sequences are usually embedded in unique DNA; thus, primers to the unique sequences can be made, and specific loci amplified enzymatically using the Polymerase Chain Reaction (PCR; Mullis *et al.*, 1986; Saiki *et al.*, 1988; White *et al.*, 1989). Alleles of various lengths, generated through mechanisms such as replication slippage (Levinson and Gutman, 1987), can be resolved on high resolution gels. Because microsatellites are typically in the range of 100 base pairs long, they are more amenable to PCR-based approaches than are minisatellites.

These simple sequences have applications for genome mapping, linkage studies, and for various population studies, as these markers are useful for identity testing and for inferring relationships (Tautz, 1989). An advantage of this PCR-based approach is that very small samples of blood or tissue can be used, even if the DNA is partially degraded, which is an important consideration in many field studies. Microsatellite analysis does, however, require considerable laboratory expertise, in terms of identifying polymorphic loci and designing PCR primer sets to them.

2. Mitochondrial variation

Mitochondrial (mt) DNA analysis has been a widely-used tool for population studies. The theory and applications of mt studies have been well reviewed (*e.g.* Wilson *et al.*, 1985;

Avise *et al.*, 1987; Moritz *et al.*, 1987; Harrison, 1989; Ovenden, 1990; Avise, 1991). Interest in this molecule for population studies was generated when it was reported that the mutation rate was estimated to be 5-10 times that of single copy nuclear DNA (Brown *et al.*, 1979; Brown *et al.*, 1982). It was also reported that the genome had stable gene content and arrangement (Wilson *et al.*, 1985), that individuals were homogeneous for mtDNA types (Wilson *et al.*, 1985), and that inheritance was matrilineal, without recombination, with very low probability of paternal leakage (Lansman *et al.*, 1983; Gyllensten *et al.*, 1985; Avise and Vrijenhoek, 1987).

As more work has been done on mtDNA, however, exceptions to these "rules" have been observed. Paternal leakage was detected in *Drosophila* (Kondo *et al.*, 1990) and mice (Gyllensten *et al.*, 1991) after several generations of backcrossing. Zouros *et al.* (1992) found extensive paternal contributions in the mussel *Mytilus*, in both inter- and intra-specific crosses. The rate of paternal contribution in the mussel was estimated to be about 1000 times that found in mice, and was detected over the span of only one generation. Another source of heteroplasmy in mtDNA is due to repeated elements in the mt genome. Length heteroplasmy has been reported for several species, including scallops, (LaRoche *et al.*, 1990), monkeys (Hayasaka *et al.*, 1991) and shrews (Stewart and Baker, 1994).

One part of the animal mitochondrial genome, the control

region (also called the D-loop), has been the focus of many mtDNA studies, as it has been reported to be the most variable portion of the mt genome, with a rate of mutation estimated to be as much as 5 times that of the rest of the mt genome (Aquadro and Greenberg, 1983). The control region contains the origin of heavy strand synthesis, and promoters for heavy and light strand transcription (Clayton, 1982, 1984). Despite the variability that has been found in the control region, there are nucleotide blocks that have been well conserved. Three conserved sequence blocks (CSBs) were identified in an alignment of mouse and human control regions (Walberg and Clayton, 1981). Since then, much attention has been given to the identification of CSBs in other species (eg. rat, Brown *et al.*, 1986; hominids, Foran *et al.*, 1988; *Xenopus*, Bogenhagen and Morvillo, 1990) and to the elucidation of the roles of these elements. Chang and Clayton (1985) reported that in humans, CSB elements are implicated in the proper transition from RNA to DNA synthesis. Bennett and Clayton (1990) showed that RNase MRP (Mitochondrial RNA Processing), an endonuclease involved in mtDNA replication, requires CSBs for efficient cleavage. A conserved control region sequence has also been suggested to be important for the functioning of a bovine endonuclease that may be necessary for mtDNA replication (Low *et al.*, 1987).

In cetaceans studied so far, there has been no evidence of size variation or heteroplasmy (*e.g.* Arnason *et al.*, 1991a;

Wada *et al.*, 1991; Baker *et al.*, 1994; McMillan and Bermingham, 1996) in the mt genome. However, the very high rate of control region evolution found in humans has not been observed in cetaceans. Hoelzel *et al.* (1991a) investigated the tempo of control region evolution in cetaceans, and reported that the rate of nucleotide substitution was an order of magnitude lower than that suggested for the human control region, but about the same as has been reported in other primates (Foran *et al.*, 1988) and rodents (Brown *et al.*, 1986). Although the mutation rate may not be as high as reported in humans, there seems to be sufficient variation in the control region for population studies, as reviewed below (sec. 2.11).

One very important consideration in choosing a molecule for genetic studies is to understand the strengths and limitations of that particular molecule. In the case of mtDNA, it seems to be an excellent marker for studies of evolution, as information that can be obliterated in recombination can be preserved in the mitochondrial genome, which does not recombine. This molecule is also useful in establishing matrilineal phylogenies and tracing the origin of migrant males in species where dispersal is through the males and females are philopatric (*e.g.* macaques, Melnick and Hoelzer, 1992; lesser snow geese, Quinn, 1992). Melnick and Hoelzer (1992), however, caution against the use of mtDNA as an all-encompassing genetic marker, as their results showed

that mtDNA variation can be a poor reflection of nuclear variation: where mtDNA variation consisted of 9% within regions and 91% among regions, the exact opposite was true for nuclear variation, as the movement of males among regions prevented differentiation of nuclear markers.

Because male and female sperm whales have drastically different ranges, they seem to fit this pattern of extensive movement of males, but limited female migrations. Caution must be exercised in interpreting genetic results, as males and females must be considered separately. Mitochondrial DNA analysis may shed light on stock divisions of females and their offspring, while telling little about the mature males. In sperm whales, then, a comparison of nuclear and mitochondrial variation should be done to gain an appreciation of all the genetic variation present in the species.

Examples of DNA techniques applied to cetaceans

Studies using molecular genetics techniques described above, often in combination with observational data, are revealing much about cetacean biology. For example, genetics studies have been used to reveal social structure, to determine paternity, to assess stock division, to examine the recovery of species post-whaling, to look at migration and mating behaviour, and to determine sex of individuals. A summary of some of these studies is presented in Table 1.1. This table is not intended to be inclusive, but rather to show the range of cetacean genetic studies that have been done.

Table 1.1: An overview of genetic studies of cetaceans

Author(s)	Year	Analysis
Southern *et al.*	1988	sequence comparison of cetacean mitochondrial genome
Stevens *et al.*	1989	RFLP analysis of whole mtDNA from captive orcas
Amos and Hoelzel	1990	fingerprinting of samples collected by biopsy darting
Baker *et al.*	1990	humpback whale population structure using mtDNA RFLPs
Amos and Hoelzel	1991	methods for long-term preservation of cetacean skin samples
Amos and Dover	1991	satellite sequences in minke whales
Baker *et al.*	1991	sexing humpbacks using a human Y chromosome-derived probe
Brown *et al.*	1991	sex determination in beluga whales using Y-specific probe
Dizon *et al.*	1991	stock structure of forms of spinner dolphins using mtDNA RFLPs
Duffield and Wells	1991	social structure in *Tursiops* using chromosome, molecular and protein studies
Hoelzel	1991	mitochondrial control region variation in orcas
Hoelzel and Dover	1991	minke whale population analysis using control region sequences and RFLPs
Hoelzel *et al.*	1991a	evolution of cetacean control region

Table 1.1 (continued)

Author(s)	Year	Analysis
Hoelzel *et al.*	1991b	paternity testing in captive orcas using fingerprinting
Schaeff *et al.*	1991	analysis of N. and S. Atlantic right whales using mtDNA RFLPs
Schlötterer *et al.*	1991	comparison of simple sequence repeats and flanking sequences in 11 cetacean species
van Pijlen *et al.*	1991	minke whale population study using multilocus fingerprinting
Wada *et al.*	1991	population analysis of minke whales using mtDNA RFLPs
Amos *et al.*	1992	usefulness of sloughed cetacean skin for genetic analysis
Milinkovitch	1992	DNA-DNA hybridizations among Delphinidae, Phocoenidae, artiodactyls, dog and man
Palsbøll *et al.*	1992.	sex determination using universal SRY and ZFX/ZFY primers
Amos *et al.*	1993	pilot whale social structure as revealed by DNA fingerprinting
Baker *et al.*	1993a	population structure of humpbacks using control region sequences
Baker *et al.*	1993b	population structure of humpbacks using DNA fingerprinting
Milinkovitch *et al.*	1993	evolution of cetacean mt 12S and 16S genes

Table 1.1 (continued)

Author(s)	Year	Analysis
Schaeff *et al.*	1993	mtDNA RFLPs of W. N. Atlantic right whales that differ in use of Bay of Fundy nursery
Baker and Palumbi	1994	PCR amplification and sequencing of control region from whale meat samples available commercially in Japan
Baker *et al.*	1994	genetic population structure of humpback whales worldwide using mtDNA RFLPs
Richard *et al.*	1994	sex determination in cetaceans using sperm whale specific SRY primers and "universal" ZFX/ZFY primers
Medrano-Gonzalez *et al.*	1995	control region sequences from humpback whales in Mexican Pacific ocean
Palsbøll *et al.*	1995	control region sequences from humpback whales representing 5 aggregations in N. Atlantic and 1 in Antarctic
McMillan and Bermingham	1996	mtDNA RFLPs in Dall's porpoise from N. Pacific

Some studies are described in more detail here to demonstrate the application of these techniques to cetaceans.

Minisatellite and microsatellite studies of cetaceans

Various studies of nuclear markers in cetaceans have been conducted to investigate questions about paternity and population structure. Hoelzel *et al.* (1991b) compared minisatellite profiles in captive orcas in a paternity assessment. Of the two potential fathers, one had a DNA fingerprint that clearly excluded him as the father, while the other male shared all paternal bands with the calf. Amos *et al.* (1993) used samples of pilot whales (*Globicephala melas*) taken in the Faeroe Island fishery, to investigate the social structure of this species. They typed each sample for 6 microsatellite loci and one minisatellite locus. As some of the samples came from presumed entire schools of pilot whales, some of which contained pregnant females, the authors had a unique sample in which to study mating and social structure. They found that although males do not disperse from their natal pods, they do not seem to mate within their pod either, as 89% of paternal alleles were unique within a cohort. The studies above involved samples from whaling and captive animals, but fingerprinting studies have also been done on free-living populations. Using biopsy samples from humpback whales (*Megaptera novaeangliae*), Baker *et al.* (1993a) analyzed three hypervariable minisatellite DNA probes. They found extensive nuclear variation with these probes, despite the

fact that these whales were hunted to near-extinction in many areas.

Mitochondrial studies applied to genetic definitions of stocks

The first mt studies in cetaceans were Restriction Fragment Length Polymorphism (RFLP) analyses of the entire mt genome. With the advent of PCR, sequencing of parts of the mt genome (especially the control region) have become more common. The goal of most of these studies is to assess population structure and examine stock boundaries. One of the criteria used in determining a biological stock is genetic differences (others being life history parameters and morphological differences). Hoelzel (1992) gives the definition of genetic stocks as genetically differentiated conspecific populations. Genetic analyses have advantages over other methods of assessing biological stocks because easily observable characters such as morphological differences (*e.g.* dorsal fin shape) or colour variation will only reflect some of the genetic variation present. Also, there may be a large environmental component to this kind of variation. The approach taken by most researchers interested in determining genetic stock boundaries for cetaceans is to begin with *a priori* classifications of populations, usually based on presumed geographic divisions or management units defined by the IWC. Genetic differences within and between populations are assessed, often with comparisons to more geographically distant populations of the same species or to other species.

If little or no genetic difference is found between management stocks, it suggests that these are not biological stocks and should not be treated separately for management; if, on the other hand, differences between management stocks in the same geographic area are similar in magnitude to differences between widely separate populations of the same species, or approaching the differences between two species, genetic stock structure is indicated.

For example, several studies of minke whales (*Balaenoptera acutorostrata*) have been carried out, as there is much controversy surrounding the resumption of hunting in this species. These studies have used the current management stocks that have been assigned by the IWC. Wada *et al.* (1991) performed an RFLP analysis of minke whale mtDNA and found no shared haplotypes between three forms of minke whales (North Pacific, Southern Hemisphere, and dwarf), indicating genetic separation between them (even though the dwarf samples were from the same geographic area as the Southern Hemisphere ones). In addition, the haplotype frequencies were very similar in two Antarctic management areas defined by the IWC, suggesting that these stock distinctions are not appropriate. Hoelzel and Dover (1991) did RFLP analyses of the mitochondrial control region of minke whales from the Antarctic, West Greenland and the Western North Pacific. They estimated the mean pairwise sequence divergence within and between populations based on the proportion of shared

restriction sites (Nei and Li, 1979). Similar to the results of Wada *et al.* (1991), this study suggests there is little or no genetic distance between two of the Antarctic management areas. However, the authors report that the genetic distance between the Northern Hemisphere populations was as large as the genetic distance reported between some odontocete species.

Baker *et al.* (1990) assessed humpback whale populations using the RFLP method. They had samples from the Pacific Ocean (Alaska, Hawaii and California) and from the Atlantic. They found that there was no overlap in haplotypes from the two oceanic populations, nor between the Pacific feeding grounds (South East Alaska and California), even though individuals from both these feeding grounds mix on the common Hawaiian wintering ground. Thus, the Alaska and Californian samples should be considered separate biological stocks.

The population structure in western North Atlantic right whales (*Eubalaena glacialis*) was evaluated by Schaeff *et al.* (1993). Only 3 different restriction fragment patterns were observed in 96 samples after digestion of the whole mt genome with 11 restriction enzymes. One mtDNA haplotype was not seen among females that brought all their calves to the Bay of Fundy (a summer and fall feeding area and "nursery" ground), although it was present in females that use other, unknown, areas at least some of the time. These results suggest that this right whale population is divided into 2 subgroups, based on utilization of the Bay of Fundy feeding area.

Hoelzel (1991) performed RFLP studies of the whole mt genome of orcas to determine whether there was any genetic differentiation between two populations off Vancouver Island. These populations, called transient and resident, are different in many aspects of behaviour, including their diets, their group size and the extent of ranging (although the ranges of the residents and transients overlap). The percent sequence difference was estimated and it was found that the difference between the resident and transient whales was about the same as the distance between either of these and samples from Atlantic orcas. This is an intriguing result, as it suggests substantial genetic differentiation between orcas coexisting in a geographical area.

In an RFLP study of spinner dolphins (*Stenella longirostris*), Dizon *et al.* (1991) examined two morphological forms (found in the eastern tropical Pacific), one geographically distant population (from the Timor Sea), plus spotted dolphins (*S. attenuata*) from the eastern tropical Pacific. Some mt haplotypes were found only in the Timor Sea samples, while others were specific to the spotted dolphins. Within the eastern tropical Pacific, no correspondence between haplotype or morphological form could be found. These forms also shared rare haplotypes. The authors note surprise in finding mtDNA exchange in these forms, given the obvious morphological differences between them.

Based on the studies done to determine stock boundaries

in cetaceans, it seems that no rules or patterns have developed. Some species may have extensive genetic dispersal, while others, like the minke, may have little exchange between oceans. Morphologically distinct spinner dolphins show evidence of genetic exchange (at least of the mt genome), indicating that morphological differences are not always indicators of genetic differentiation. The finding that orcas off Vancouver Island might be as genetically distinct as orcas from different oceans has important implications for conservation. Humpback whales that mix on common wintering grounds can be unambiguously assigned to feeding grounds; this shows the need to consider migration and site fidelity when describing population structure. Perhaps broader conclusions will be evident when even more studies of cetacean genetics are done. Such studies are occurring; most of these are placing more emphasis on examining changes at the DNA level through sequencing, and putting less emphasis on restriction endonuclease (RE) studies.

Cetacean control region studies

Not many years ago, sequencing of DNA (Sanger *et al.*, 1977) was dismissed by some authors as too costly and time-consuming for population work (*e.g.* Hoelzel and Dover, 1989); at present, however, it has become a feasible alternative to RFLP work. It is interesting to see how the results of the two methods compare, and this comparison is facilitated by the fact that some of these studies have been repeated using

direct sequencing of the mitochondrial control region in the place of RFLP studies. For example, Baker *et al.* (1993b) compared observed nucleotide diversity in humpback whales using both RFLP and sequence methods and reported that the diversity found by RFLP analysis was about ten-fold lower than that found by sequencing. However, the phylogenetic relationships between the unique control region sequences were identical in topology to those obtained by parsimony analysis of whole mtDNA RFLPs performed in the earlier study. Hoelzel and Dover (1991) used sequence data from the control region "to maximize the degree of possible discrimination between genetic stocks" of the minke whale. These sequencing results confirmed RFLP results, in that two Antarctic management "stocks" had very low genetic distance. Spinner dolphin variation was re-analyzed using control region sequencing, and the earlier (RFLP) conclusion that recent or ongoing mtDNA exchange between stocks exists was supported by sequence data (Garcia and Dizon, 1993). It is likely that more studies of stock discrimination will be performed using sequencing rather than RFLPs. Such studies are facilitated by conserved mitochondrial DNA primers (control region, cytochrome b, 12S rRNA; Kocher *et al.*, 1989). Palumbi *et al.* (1991) seem decidedly on the side of sequencing, and state that "sequence data are intrinsically better" than RFLP data, because of ambiguity in interpreting mutation differences in a RE site and because of the better resolution of sequence data over

RFLP data. For reasons discussed in chapter 2, it is this approach -- sequencing of the mitochondrial control region -- that was employed in my analysis of sperm whale genetics.

Contribution of this thesis

As sperm whales are distinct among the cetaceans in terms of evolution, social organization and population structure, there are many interesting questions that genetic analyses of this species can address. In this thesis, I demonstrate that the mtDNA control region can be amplified from sloughed sperm whale skin and sequenced (chapter 2), and I use these sequences to gain insights into molecular evolution of the control region (chapter 3), social organization of groups of female and immature whales (chapter 4), and population structure within and among 3 major ocean basins (chapter 5).

CHAPTER 2: PCR amplification of the control region from sloughed skin and methods for assessing its variability in sperm whales

Introduction

Genetic studies of cetaceans have examined a wide range of questions, including stock boundaries, social structure, paternity assessment and conservation issues (reviewed in Chapter 1). These studies have used a variety of different approaches including allozymes, minisatellite DNA, microsatellite DNA and mtDNA. The particular genetic approach used depends largely upon the question being addressed, as each method has strengths and weaknesses.

Mitochondrial DNA has been used for numerous population studies. The mtDNA from animals has many advantages for this work, including a rapid rate of evolution (compared to single copy nuclear DNA), a compact genome with a uniform gene content and arrangement, maternal inheritance with lack of recombination (see chapter 1). This molecule is particularly useful when examining genetic differentiation in species where males move freely among populations, obscuring nuclear DNA differentiation, while females have restricted ranges, leading to differentiation of the mtDNA molecule. As these patterns of male and female movement have been observed in sperm whales, mtDNA seemed to be an appropriate marker for analysis of population differentiation of female and immature sperm

whales. The focus of this work was the mtDNA control region, which is the major non-coding region in the genome, and is expected to accumulate mutations more quickly than the rest of the genome.

Most genetic studies of cetaceans have used biopsy darting, a process that gives sufficient tissue to enable extraction of mtDNA molecules. The work in this thesis, on the other hand, relied on collection of sloughed skin, and the samples were often minute. Therefore, a PCR-based approach to assessing genetic variation in sperm whales was required.

This chapter describes the collection and handling of sloughed sperm whale skin and the laboratory procedures used to (1) determine that it was sperm whale mtDNA control region, and not contaminating DNA, that was being amplified; and (2) assay the control region for variability.

Materials and methods

2.1 Collection of sperm whale samples

I participated in field work and collected skin samples as a source of DNA (Whitehead *et al.*, 1990; Amos *et al.*, 1992) in work carried out off of mainland Ecuador (3°S-2°N, 80-83°W), and the Galápagos Islands, Ecuador, (2°S-1°N, 89-93°W) between January and April, 1991. Additional samples were available from the Galápagos in 1989, the South Pacific (1992-1993), and the N. Atlantic (1993). I was also given sperm whale samples from Prince Edward Island (from Pierre-Yves Daoust, Atlantic Veterinary College), Nova Scotia (from the Nova Scotia

Stranding Network), the Seychelles (from Benjamin Kahn, Dalhousie University), the Azores (from Jonathan Gordon, International Fund for Animal Welfare) and the Canary Islands (Michel André, Centro de Mamiferos Marinos de Canarias). The Prince Edward Island and Nova Scotia samples were from stranded whales, while the other samples were collected non-invasively from free-living whales, as described below. Additionally, samples from other cetaceans were available from stranded animals. Dr. Jon Lien (Memorial University of Newfoundland) provided the pygmy sperm whale sample (*Kogia breviceps*), and the Nova Scotia Stranding Network donated samples from northern bottlenose whale, Atlantic white-sided dolphin, pilot whale and humpback whale.

Field work was conducted from a sail boat. Sloughed skin was collected in the wake of swimming whales or in the "slick" left behind when the whale began a deep dive. Skin was usually collected from the deck using a dip net, although snorkellers with hand nets were also employed. Details of sample number, local time, position, and number of possible whales from which this sample could have come, were recorded in the field. Skin samples coming from lone whales for which identification photographs were taken, were clearly noted and reference was made to the photograph number; we have observed that sloughed skin sinks quickly, and in such cases, the tissue sample can be linked to the identified whale. While at sea, samples were preserved in a 20% dimethyl sulphoxide

(DMSO) NaCl-saturated solution. Care was taken to thoroughly clean dip nets between samples, and all skin was handled with forceps to reduce the possibility of human contamination or degradation of the samples. After the completion of the field season, samples were frozen at -20°C. Samples from stranded whales were kept on ice during shipping and frozen as soon as possible.

DNA was extracted from skin by grinding skin in liquid nitrogen, digesting the sample with proteinase K, and organically extracting the DNA (Amos and Hoelzel, 1991), and by Chelex extractions, where the skin is boiled in a solution of 5% chelating resin (BioRad) and used directly as template in PCR reactions (Walsh *et al.*, 1991).

2.2 PCR amplification of the mtDNA control region

The control region of the mitochondrial genome from sperm whales and other cetaceans was amplified using PCR (Mullis *et al.*, 1986). Initially, I used the "universal" primers described by Kocher *et al.* (1989), but to increase stringency, I designed "cetacean-specific" primers. These primers were based on the published orca sequence (Hoelzel, 1989) to which I added *Eco*RI recognition sites. One primer annealed to $tRNA^{Thr}$ (5'-TCACCGGTGAATTCCCCGGTCTTGTAAACC-3') while the other annealed to $tRNA^{Phe}$ (5'-CAGAATTGGAATTCATTTTCAGTGTCTTGCTTT-3'). Reaction conditions were optimized for annealing temperature, $MgCl_2$ concentrations, and primer and template amounts. PCR reactions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM

$MgCl_2$, 200 μM each dNTP, 0.5 μM of each primer, and 0.625-0.5 Units of Taq polymerase, and were done in a volumes of 25 to 100 μL, depending on what subsequent reactions were to be carried out. Template amount was 1-4 ng of organically-extracted sample or 71.5 % v/v of Chelex-extracted samples. Thirty cycles were completed, consisting of denaturation at 94°C for 1 minute, annealing of primers at 50-52°C for 1 minute and extension at 72°C for 1 minute. All PCR amplification experiments included controls in which no DNA was added. Products were size fractionated on 1% agarose gels (1 X TAE buffer) at 30-40 V for approximately 18 h, stained in ethidium bromide (0.5 μg/mL) and visualised under UV light.

2.3 Cloning procedures

2.3.1 *RE digestion of PCR products*

PCR products were digested with *Eco*RI to produce control region molecules with ends complementary to those of the cloning vehicle, M13 mp18 (Messing, 1983), which was digested with *Eco*RI and treated with bacterial alkaline phosphatase (Pharmacia). Ligations were carried out at 14°C overnight. DNA was purified before transfection by binding to glass beads and washing (GeneClean, Bio 101).

2.3.ii *Preparation of competent cells*

Competent cells were prepared by inoculating 50 mL of 2 x YT broth (16 % w/v bacto-tryptone, 10% w/v bacto-yeast extract, 5% w/v NaCl, pH 7.0; Sambrook *et al.*, 1989) with 0.5 mL overnight TG1 culture. This was incubated with shaking

(250 rpm) at 37°C until OD_{600} = 0.35 - 0.6 (approximately 2 h). The culture was divided into 2 tubes and subjected to centrifugation in a JA20 rotor (Beckman) for 5 min. with no brake. The supernatant was discarded and the pellets were gently resuspended in approximately 5 mL ice cold 50mM $CaCl_2$. The pellets were combined and brought up to a volume of 35 mL with $CaCl_2$. This was left on ice overnight; the supernatant was carefully siphoned off leaving the competent cells behind in about 4 mL $CaCl_2$. 200 μL of competent cells were used for each transfection reaction. An aliquot of the ligation reaction was added to the competent cells and incubated on ice for at least 40 min. The competent cells/DNA were heated to 42°C for 5 min. and then left at room temperature until plating. All transfection experiments included M13 RF (replicative form) DNA and no DNA controls.

2.3.iii *Plating of transfection reactions and plaque selection*

2 x YT plates were prewarmed for at least one hour. Each transfection reaction was combined with 3 mL top agar, 80 uL of 2% X-gal (in DMF) and 25 μL 2.5% IPTG. The top agar mixture was allowed to harden for 15 min. and was incubated at 37°C overnight (approximately 16 hours). Positive (clear) plaques were picked with a Pasteur pipet and placed in 1 mL of 2 x YT broth with a drop of chloroform. Plaques were left at room temperature for at least one hour and then stored 4°C.

2.3.iv *Preparation of single and double-stranded M13 clones*

Single-stranded bacteriophage and double-stranded RF M13 DNA was prepared using mini-prep procedures. Three mL 2 x YT were inoculated with 75 μL overnight TG1 culture and 150 μL of plaque stock, and were grown for 5-6 h at 37°C with agitation. This was centrifuged for 5 min. RF DNA was isolated from the bacterial pellet by lysis with SDS and NaOH as described by Sambrook *et al.* (1989). Bacteriophage particles were precipitated with PEG and phenol/chloroform extracted (Sambrook *et al.*, 1989).

2.4 RE digestions of the control region

Control regions from 13 geographically-separated sperm whale samples (Ecuador, Galápagos, Seychelles, Azores and PEI) were digested with 16 REs (Table 2.1, p. 45), surveying just over 9% of the control region. In addition, the pygmy sperm whale control region was digested with 11 of the enzymes used for the sperm whale (Table 2.1, p. 45). Products were size fractionated on 1-2% agarose gels.

2.5 Deletion analysis

To determine the sperm whale control region sequence, a set of nested deletions was generated by exonuclease III/mung bean nuclease digestions (Henikoff, 1984). M13 RF DNA was digested with PstI (3' overhang) and XbaI (5' overhang). Exo III digestion was carried out such that aliquots of the reaction were removed every 30s; this was estimated to be the time for 200 nucleotides to be removed. This resulted in

insert control region DNA with between 200 and 800 bp removed.

2.6 Dideoxy sequencing of cloned control regions

DNA from single-stranded and RF of M13 were sequenced by the dideoxy method (Sanger *et al.*, 1977), using alpha-^{35}S-dATP (1000Ci/mmol) and the T7 DNA polymerase (Pharmacia). The deletion series resulted in the sequence of one strand of the sperm whale control region. From this sequence, synthetic oligonucleotides were designed for double-stranded sequencing of the complementary strand. The tRNAs adjacent to the control region ($tRNA^{Pro}$ and $tRNA^{Phe}$) were sequenced and thus defined the beginning and end points of the control region.

For sequencing subsequent sperm whale control regions, synthetic oligonucleotide primers were used, based on the original sperm whale sequence. These primers annealed to block "a", block "c", block "F", block "b" block "D" and block "F" (chapter 3; Figure 3.1).

Sequencing reactions were resolved on 6-8% polyacrylamide gels with 7.8M urea, and 1X TBE or 1X gel-tolerant buffer (containing taurine instead of borate). Gels were run with a constant power of 50 W for 2-6 h. Gels were exposed to X-ray film (Kodak X-omat AR) for 12 h to 3 days.

2.7 Direct sequencing of PCR products

In addition to sequencing of cloned control regions, I tried direct sequencing of PCR products. Three methods were evaluated. (1) Asymmetric PCR amplifications. Single-stranded PCR products were produced by asymmetric

amplification of the control region. The first round of amplification proceeded as described above. Templates for asymmetric re-amplification were aliquots of the double-stranded PCR reaction or gel slices excised from agarose gels. One primer was used in limiting concentrations (0.05-0.2 μM), while the other was at 1μM. Various combinations of limiting primers were tried.

(2) ds sequencing using GeneClean purification. Alternatively, direct, double-stranded sequencing of the PCR products was tested. The best sequence was obtained when the entire PCR product was treated with GeneClean to remove excess primers and unincorporated nucleotides (this was superior to gel isolation of fragments). Primers were then annealed to the control region by boiling primer, template, annealing buffer and DMSO for 5 minutes, followed by snap cooling on wet or dry ice. Sequencing was then carried out as above.

(3) ds sequencing using exonuclease I / shrimp alkaline phosphatase (exoI/sAP). PCR products were purified prior to sequencing using the method of Werle *et al.* (1994). This process purifies the PCR reaction by removing excess PCR primers (using exonuclease I) and unincorporated dNTPs (using shrimp alkaline phosphatase) which are thought to interfere with subsequent sequencing reactions. After incubation of an aliquot of the PCR reaction with exoI/sAP, dideoxy sequencing with the T7 polymerase was carried out as described above.

Results and discussion

2.8 Amplification of mtDNA control region from sperm whales

I have demonstrated that the mtDNA control region from sperm whales can be reliably PCR amplified from sloughed skin samples, by comparing these products to those from known sperm whale tissue (from stranded animals). In addition, sperm whale PCR products were compared to those from other cetaceans (pygmy sperm whale, northern bottlenose whale, pilot whale, humpback whale, and dolphin). All the sperm whale products were the same size, approximately 1100 bp, which was about 50 bp larger than those for the other cetaceans (Fig. 2.1).

Hal Whitehead and colleagues have considerable experience collecting sloughed sperm whale skin at sea, and because it is observed to sink quickly, one can be certain that the collected tissue came from the nearby sperm whales. However, it was still necessary to erase any possible doubts about what is being scooped out of the sea by comparing PCR products of known sperm whales to those from sloughed skin thought to be derived from sperm whales. Additionally, by observing that sperm whale products are larger than those of other cetacean species, the possibility of accidentally amplifying the control region from another cetacean is eliminated.

With PCR, there is always a concern that human DNA will be amplified, owing to contamination during the handling of the samples. Through the process by which I optimized PCR conditions, and by use of cetacean-specific primers (sec.

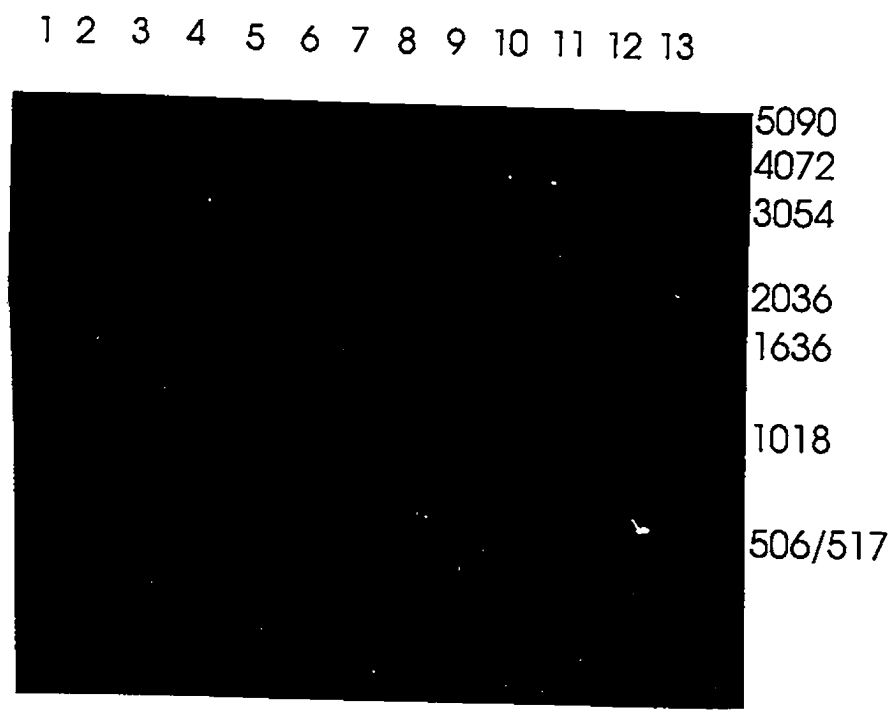


Figure 2.1: PCR amplification of the mitochondrial control region, using cetacean-specific primers. Amplified tissue was from: Lanes 1 and 2 -- stranded sperm whales; Lanes 3 to 6 -- sloughed sperm whale skin (from Azores, Seychelles Galapagos, and S. Pacific, respectively); 7 -- pygmy sperm whale; 8 -- Northern bottlenose whale; 9 -- Atlantic white-sided dolphin; 10 -- pilot whale; 11 -- humpback whale; 12 -- cow; 13 -- human. PCR products were size-fractionated on a 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Molecular size markers are the 1 kilobase pair ladder (Bethesda Research Laboratory), for which sizes are given.

2.2), I am confident that I amplified sperm whale DNA and not contaminating human DNA, as human DNA does not produce a product of the expected size for sperm whales (Fig. 2.1, lane 13). In addition, the amplification product from a cow (bovines and cetaceans share a common ancestor, discussed in Chapter 3) is not of the size expected for sperm whales (Fig. 2.1 lane 12), further demonstrating confidence in sperm whale control region amplifications.

2.9 RE digestions of the control region

Digestion of amplified sperm whale control region sequences surveyed over 9% of the region, but failed to show any variation among whales from Ecuador, Galápagos, Seychelles, Azores and PEI. Despite this, such an analysis was valuable in that the presence or absence of RE sites and the sizes of the resulting restriction fragments (Table 2.1) were consistent with the position of these RE recognition sites in the first sperm whale control region sequenced. Furthermore, the comparison to the pygmy sperm whale control region provided additional evidence that presumed sloughed sperm whale skin is indeed from sperm whale, and not contamination from another cetacean species; even a closely-related species, the pygmy sperm whale, differed from the sperm whale at 6 of 11 RE sites.

This limited RFLP analysis of the control region showed insufficient variability to enable me to carry out population analyses of sperm whales, and therefore, sequencing the

Table 2.1: Results of restriction endonuclease digestions of the sperm whale control region. Samples were Galápagos (N=3), Ecuador (N=1), Azores (N=3), Prince Edward Island (N=4) and Seychelles (N=2). Restriction enzymes also tested on the pygmy sperm whale control region are indicated (*). Enzymes that gave a different pattern with the pygmy sperm whale than with the sperm whale are shown (D).

Restriction enzyme	recognition site (5'→3')	pygmy sperm tested?	expected fragment sizes (bp)	estimated size of observed fragments (bp)
*Ava*II	GPyCGPuG	* (D)	595, 335, 145	600, 400, 100
*Bam*HI	GGATCC		none	none
*Bcl*I	TGATCA	* (D)	660, 415	700, 450
*Bgl*I	GCCNNNNNGGC	*	700, 375	750, 400
*Bst*BI	GATNNNNATC	*	none	none
*Dde*I	CTNAG		451, 186, 153, 134, 63, 63, 25	450, 200, 150 + unresolved bands
*Eco*RI	GAATTC	*	none	none
*Hha*I	GCGC	* (D)	565, 510	580, 520
*Hind*III	AAGCTT		none	none
*Hpa*II	CCGG	* (D)	740, 335	750, 350
*Mvn*I	CGCG		645, 430	600, 400
*Sac*I	GAGCTC	* (D)	685, 390	650, 450
*Sma*I	CCCGGG		none	none
*Ssp*I	AATATT		865, 210	900, 250
*Sty*I	CC(AT)(AT)GG	*	518, 492, 65	500, 500 (doublet)
*Xho*I	CTCGAG	* (D)	550, 525	550, 540

control region was necessary.

2.10 Methods used to sequence the control region from sperm whales

To find variability the control region from sperm whales, I used sequencing. In this way, many more bp are surveyed than in RFLP studies. Cloning PCR amplified products was a relatively easy process, facilitated by having RE recognition sites attached to the PCR primers, and the sequence produced was very clear and readable (Fig. 2.2). However, there is a certain error rate associated with PCR, such that the *Taq* polymerase may add an incorrect nucleotide to the growing strand. If this strand were cloned, the resulting sequence would have an incorrect bp. In my sequencing of members of well-defined groups for which we also had microsatellite data, I discovered some errors in my cloned sequences. These occurred when 2 samples thought to be the same individual based on microsatellite assays had different mtDNA haplotypes. Resequencing these samples revealed that a PCR strand with an error was cloned (as opposed to having 2 individuals with exactly the same microsatellite profiles). This finding was sufficient to convince me that direct sequencing of PCR products would be preferred to cloning. I therefore abandoned the cloning approach and made no effort to systematically calculate the error rate I encountered. I was, however, able to estimate that if the results I obtained were representative of a larger sample, the error rate associated with my PCR

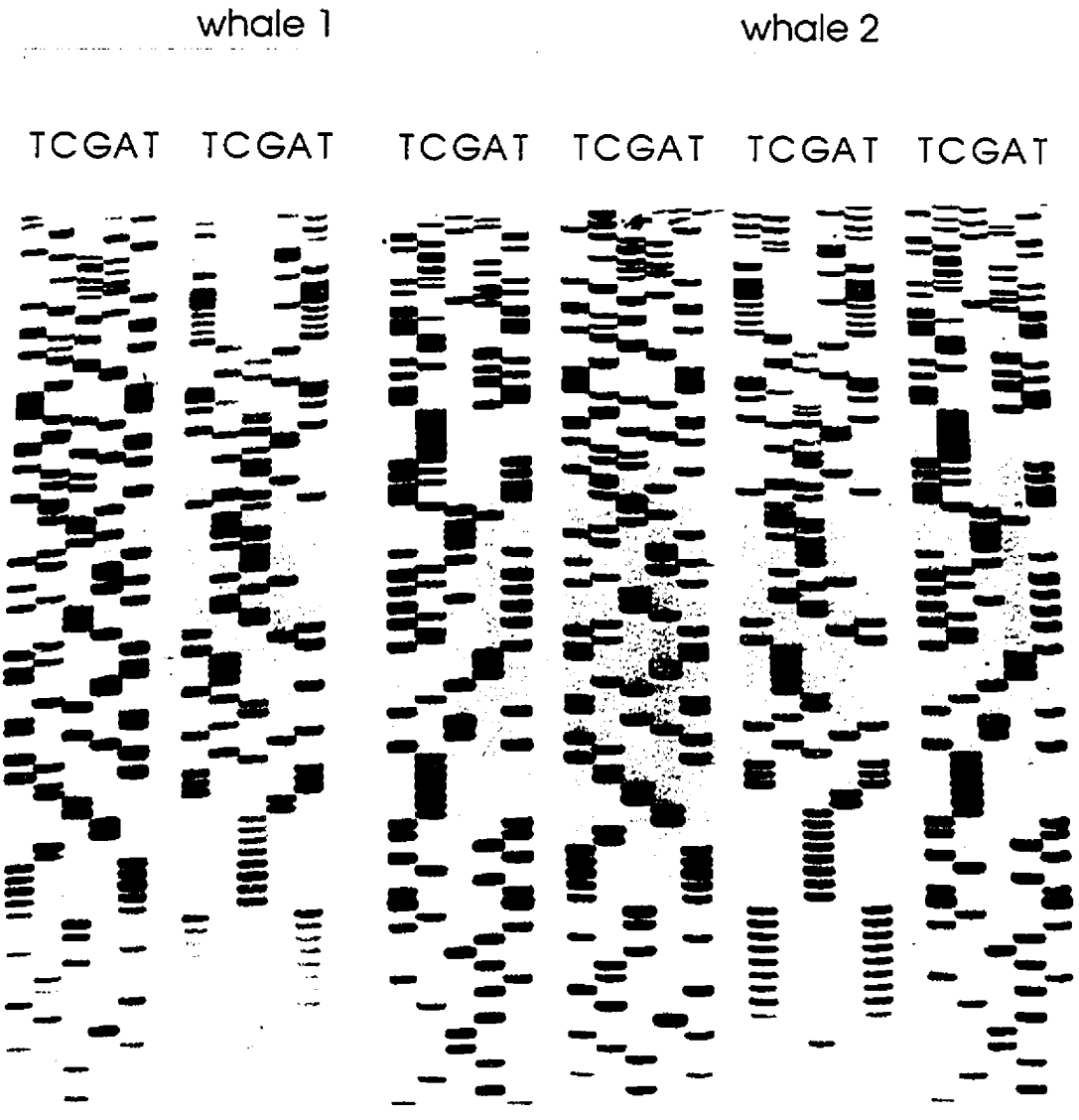


Figure 2.2: Quality of sequence obtained from cloned PCR products. Samples are control region amplifications from 2 different sperm whales, each sequenced with 3 different primers. The sequence for each sample consists of blocks of 5 lanes: TCGAT (*i*.e. the first and last lanes are the same in each set).

experiments was about 0.18% (by comparing cloned sequence to direct sequence). This means that in 500-600 bp of control region sequence, every second cloned sample would be inaccurate, greatly compromising the validity of the results. This seems very high, given that *Taq* polymerase has been described as a high fidelity, highly accurate enzyme (Eckert and Kunkel, 1990). However, Loewen and Switala (1995) have reported that the error rate associated with this enzyme can be very high. In one segment of an intergenic spacer from *Escherichia coli*, 32 out of 34 selected clones had three or more nucleotide changes from the expected sequences. These authors speculate that secondary structure associated with this region may interfere with proper elongation of the strand being synthesized by *Taq* polymerase. Given that there is secondary structure associated with the cetacean control region (Hoelzel *et al.*, 1991), the error rates I observed may not be unusual. I do emphasize, though, that the sample I used to estimate the error rate in sperm whale control region sequences was small, and may not be an accurate reflection of the actual error rate.

2.11 Direct sequencing of PCR products

The finding of errors in sequences of cloned PCR products demonstrated the need for a method to directly sequence PCR products. This effectively pools PCR products, so that one strand with an error will be greatly outnumbered by correct strands, producing accurate sequences. I tested 3 of the most

common approaches to direct sequencing reported in the literature. The first method I tried involved producing a single-stranded product by asymmetric PCR, where one PCR primer is limiting. This resulted in very clear sequence and seemed to be a promising technique. However, this method was not reliable and could not be carried out consistently.

Double-stranded sequencing proved more successful. A method that purified the PCR product by binding it to glass beads to remove excess primers and nucleotides produced sequence that was readable, but with much background.

A better approach to cleaning PCR products prior to sequencing involved exoI/sAP digestions. This was the quickest method for producing good quality sequence (Fig. 2.3) and was used for all population analyses in this thesis.

Most variability in the control region was found at the 5' end between bp 156 and 423. This is described fully in chapters 4 and 5.

Summary

Mitochondrial control region sequences from sperm whales can be reliably and confidently PCR amplified from sloughed skin. The RFLP analysis reported in this chapter did not find any variability in the sperm whale control region, and therefore, sequencing was needed to examine variability. The exoI/sAP method for purification of PCR products was found to be the fastest and easiest approach to producing good quality sequences.

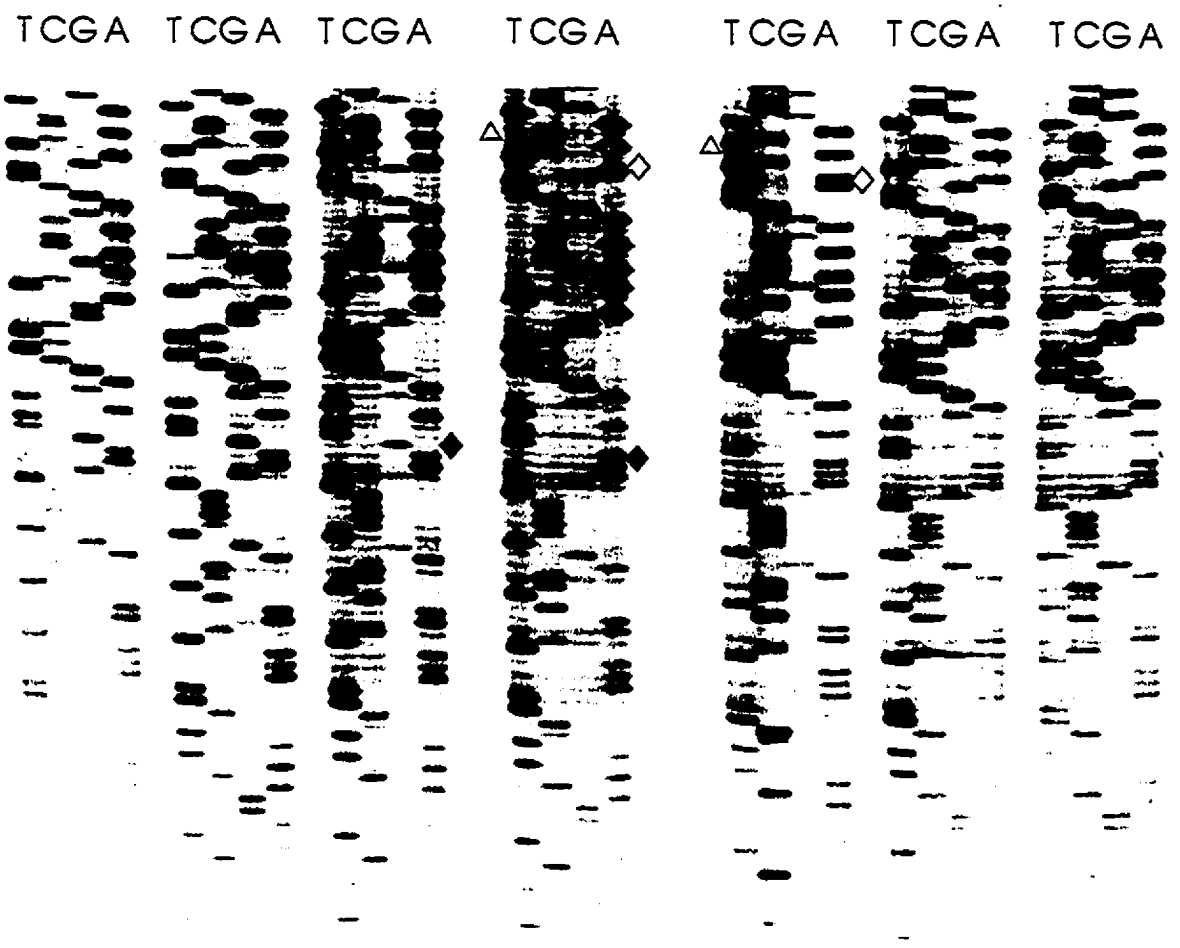


Figure 2.3: Quality of sequence obtained by direct sequencing of PCR products after exonuclease I / shrimp alkaline phosphatase purification (see text). Sequences for 7 different sperm whales are shown. The same primer was used for each. The sequence for each sample is in blocks of 4 lanes: TCGA. Examples of nucleotide substitutions are shown: G - A transitions (◆ and ◇), T - C transition (△).

CHAPTER 3: Nucleotide sequence of the control region of the sperm whale mitochondrial genome

Introduction

Of the various DNA sequences that can be used to determine genetic differences between individuals, one of the most attractive to population and evolutionary biologists is that of mtDNA. As reviewed in chapter 1, important features of the vertebrate mitochondrial genome include the high mutation rate, its stable gene content and arrangement, the homogeneity of mtDNA types within individuals, and the matrilineal inheritance with very low probability of paternal leakage. One part of the animal mitochondrial genome, the control region, the focus of many mtDNA studies, is the most variable portion of the mitochondrial genome, with a rate of mutation estimated to be as much as 5 times that of the rest of the mitochondrial genome (Aquadro and Greenberg, 1983). Within this highly divergent sequence, however, there are nucleotide blocks that are well conserved. Three conserved sequence blocks (CSBs) were identified in an alignment of mouse and human control regions (Walberg and Clayton, 1981). More recently, much attention has been given to the identification of CSBs in other species (eg. rat, Brown *et al.*, 1986; hominids, Foran *et al.*, 1988; *Xenopus*, Bogenhagen and Morvillo, 1990) and to the elucidation of the roles of these elements in the replication and expression of the mtDNA

genome. Chang and Clayton (1985) suggested that in humans, CSB elements are involved in the correct transition from RNA to DNA synthesis. Bennett and Clayton (1990) showed that RNase MRP (Mitochondrial RNA Processing), an endonuclease involved in mtDNA replication, requires CSBs for efficient cleavage. A conserved control region sequence has also been suggested to be important for the functioning of a bovine endonuclease that may be necessary for mtDNA replication (Low *et al.*, 1987).

An initial survey of control region sequences in cetaceans showed that this region has been well conserved, especially in the central, or core, area (Southern *et al.*, 1988; Hoelzel *et al.*, 1991; Arnason *et al.*, 1991b). In this chapter I compare the control region sequence from the sperm whale to that of other cetaceans. Examination of the sperm whale sequence is of interest because the sperm whale is an outlier among odontocetes (toothed whales). The sperm whale possesses distinct morphological features, such as the spermaceti organ, and with the pygmy sperm whales (*Kogia* spp.) shares a karyotype unique among cetaceans (Arnason and Benirschke, 1973; Arnason, 1974). At present there is a controversy regarding the evolutionary position of sperm whales. Milinkovitch *et al.* (1993; 1994) challenge the traditional classification of cetaceans into two monophyletic groups (the toothed whales and the baleen whales), and hypothesize that the sperm whales are more closely related to

the baleen whales than they are to any other group of toothed whales. Arnason and Gullberg (1994) also question the traditional classification, but they assert that it is the dolphins (not the sperm whales) that are the sister group of the baleen whales. The issue remains unresolved.

In this chapter, I present the control region sequence of the sperm whale and compare it to published sequences for orca and minke whale (Hoelzel *et al.*, 1991), Commerson's dolphin (*Cephalorhynchus commersonii;* Southern *et al.*, 1988), fin whale (Arnason *et al.*, 1991b) and cow (*Bos taurus;* Anderson *et al.*, 1982). The bovine sequence is a suitable comparison given that evidence provided by fossil records (Gingerich *et al.*, 1983; Barnes *et al.*, 1985; Mitchell, 1989; Gingerich *et al.*, 1990) and DNA hybridization studies (Milinkovitch, 1992) indicate a common ancestry for ungulates and cetaceans. While previous studies of cetacean control regions have shown much conservation of sequence, my work shows less conservation between the sperm whale and other cetacean control region sequences.

Materials and methods

3.1 Collection of sperm whale samples

The skin sample used in for this analysis was collected off the Galápagos Islands, Ecuador, (2°S-1°N, 89-93°W) in 1989 and was handled and stored as described in sec. 2.1.
DNA was organically-extracted from skin by the method of Amos and Hoelzel (1991).

3.2 PCR amplification of the control region, cloning and sequencing

The control region of the mitochondrial genome from sperm whale was amplified by PCR using orca primers (Hoelzel, 1989) with *Eco*RI recognition sites attached. DNA from single-stranded and replicative forms of M13 were sequenced by the dideoxy method (Sanger *et al.*, 1977). A set of nested deletions was generated by exonuclease III/mung bean nuclease digestions (Henikoff, 1984) and the sequence for one whale was determined from at least two deletion clones for each section of the control region. The sequence of the complementary strand was determined from double-stranded DNA using 17-mer synthetic oligonucleotide primers complementary to sequences of the control region (sec. 2.6). The tRNAs adjacent to the control region ($tRNA^{Pro}$ and $tRNA^{Phe}$) were sequenced and thus defined the beginning and end points of the control region. A full description of cloning and sequencing methods is given in sections 2.3 and 2.6.

3.3 Sequence alignment

The sperm whale control region sequence was aligned with sequences from other cetaceans and an outgroup (cow) using the microcomputer multiple sequence alignment program CLUSTAL V (Higgins and Sharp, 1988), with different sets of alignment parameters examined.

Results and discussion

The sperm whale control region was sequenced and the

size of this region, excluding the adjacent tRNAs, was 953 bp.

An alignment of cetacean and bovine control region sequences is shown in Fig. 3.1. An analysis of conserved sequences of the control region is shown in Fig. 3.2. The exact sequence alignment obtained depends upon the choice of alignment parameters. The parameters used to produce Fig. 3.1 were floating gap penalty = 10, fixed gap penalty = 10, pairwise gap penalty = 5, and *k*-tuple = 2 (CLUSTAL V; Higgins and Sharp, 1988). An alternative alignment was also examined (Fig. 3.3), where all gaps were given a higher penalty (fixed and floating gap penalty = 15, pairwise gap penalty = 10, *k*-tuple = 2). In this alignment, some blocks show higher sequence similarity across taxa (*e.g.* block D) while others decrease in similarity (*e.g.* block a). All of the following analysis is based on the alignment shown in Fig. 3.1. Although some sequences previously identified as conserved blocks are not well-conserved in sperm whale, they have been included here in order to facilitate comparison with previously published work.

Block F is conserved in all six species; only the fin whale exhibits one nucleotide substitution. In the alignment presented by Southern *et al.*, (1988), this block is also well-conserved in dolphin, cow, mouse and human, with 75-100% similarity to the dolphin sequence. The conservation of this region suggests that it may have an important function in mtDNA. Southern *et al.* (1988) also reported that blocks B,C,D

Figure 3.1:

Comparison of the mitochondrial control region sequence from sperm whale (GenBank Accession number M93154) to published sequences from dolphin (Southern *et al.*, 1988), orca and minke whale (Hoelzel *et al.*, 1991), fin whale (Arnason *et al.*, 1991) and cow (Anderson *et al.*, 1982). The sequence shown is the light strand in a 5' to 3' direction between $tRNA^{Pro}$ and $tRNA^{Phe}$. Dashed lines indicate insertions/deletions. Conserved blocks A through M previously identified in the literature are indicated. Blocks a, b, and c are those identified in this chapter. Arrows denote the central region.

Fig. 3.1

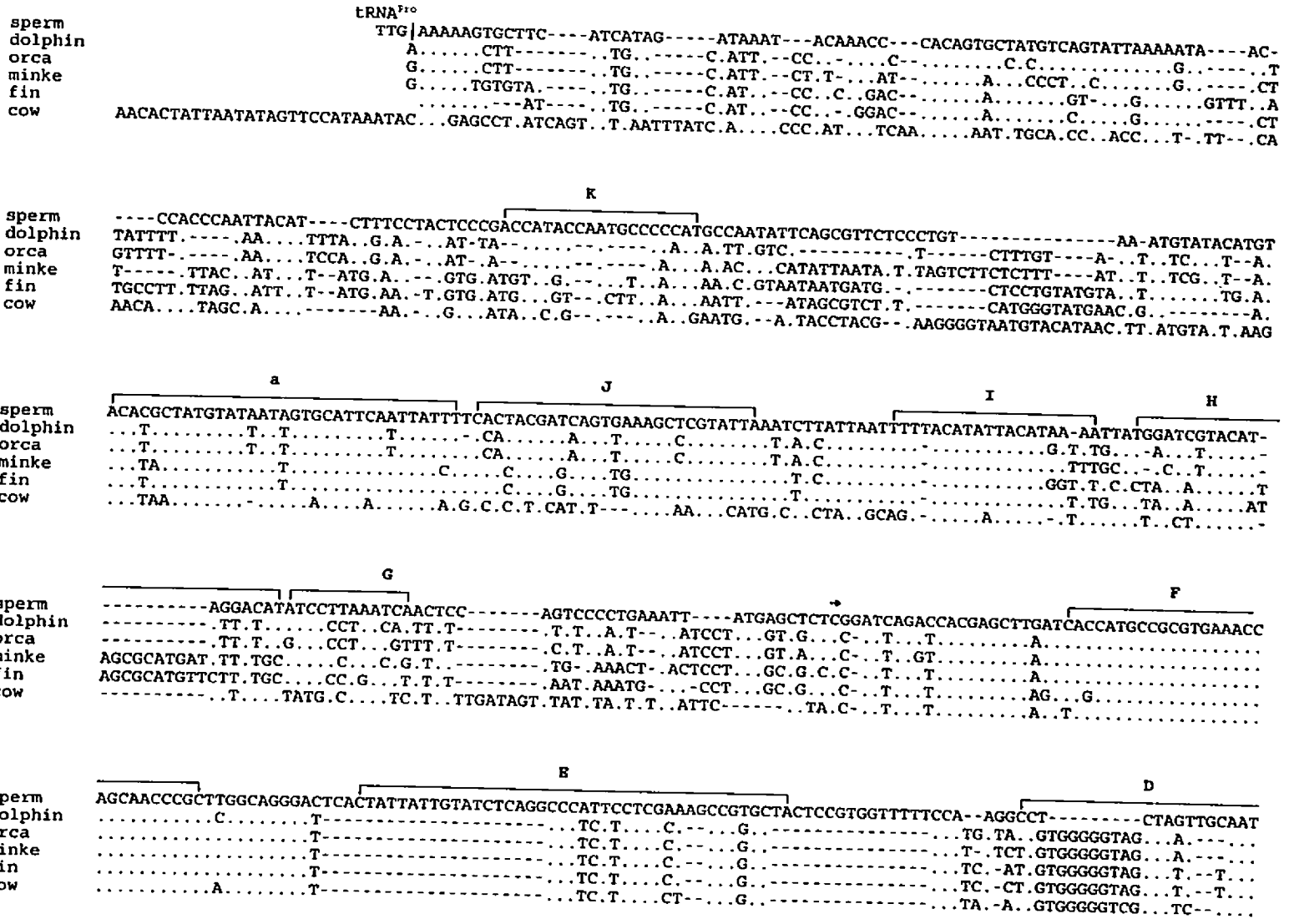


Fig 3.1 (continued)

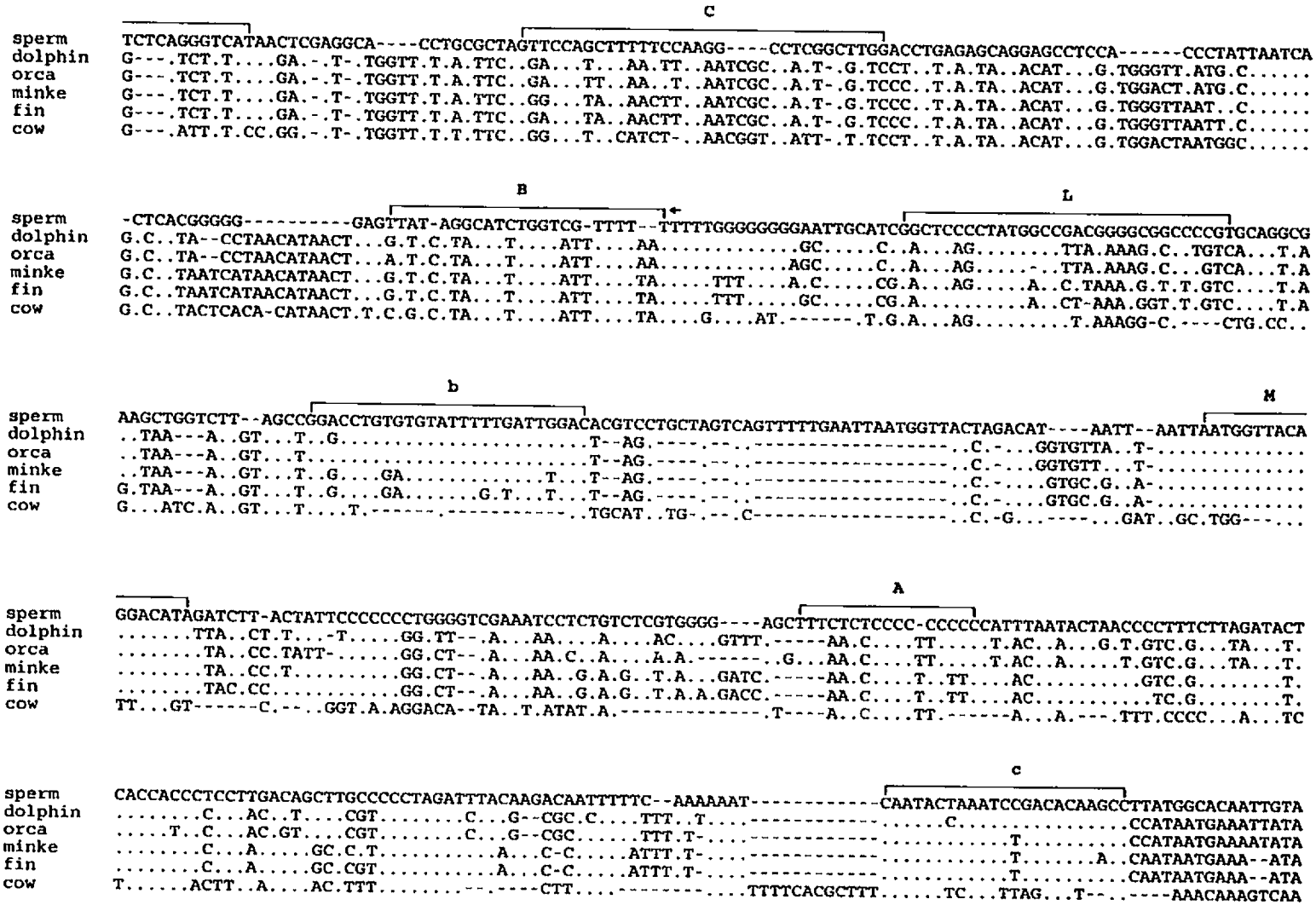
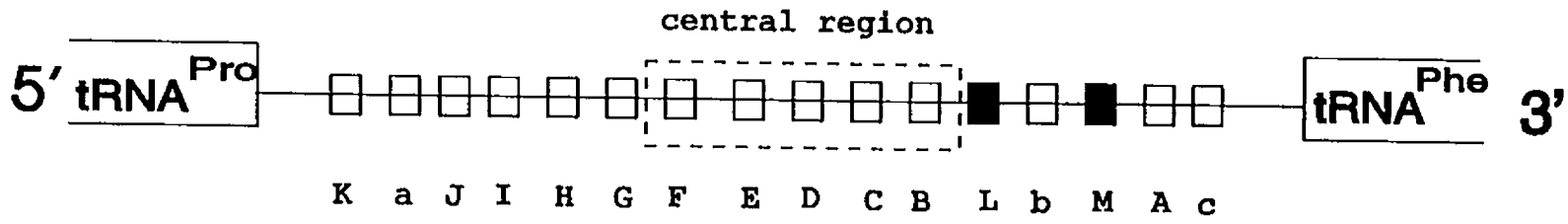


Fig. 3.1 (continued)

```
                                                    tRNA^Phe
sperm       CG---AACATTTTTTCGT---ATATAATTGG|GTT
dolphin     CAAATAATTTTATACT---CCACAA
orca        CAAATAATTTTCTGCC---CCCCTCCCCC
minke       CATG-AACATCATTCCTATTTACATAC
fin         CATG-AACGCCATCCCTATCCA-ATAC
cow         TATATAAACGCAGGCC---CCCCCCCCCC
```



dolphin	44	88	81	85	81	53	100	37	42	38	63	55	96	100	48	96
orca	44	88	81	85	81	47	100	37	42	41	63	55	100	100	71	96
minke	48	88	85	80	35	73	100	36	42	41	63	55	85	100	43	91
fin	48	94	85	90	32	63	96	36	42	41	63	59	77	100	43	96
cow	48	76	46	65	87	42	100	36	42	41	63	55	31	94	38	65

mammalian CSBs 1 2

Figure 3.2: Identification of conserved sequence blocks resulting from alignment of sperm whale, dolphin, orca, minke whale, fin whale and cow. Alignment is in the 5' to 3' direction between $tRNA^{Pro}$ and $tRNA^{Phe}$. Blocks represented by upper-case letters were described in the literature previously. Blocks a, b, and c are those resulting from the alignment with sperm whale and were identified in this chapter. Solid blocks are those found only in bovine and cetacean sequences. The values beneath each box represent the percentage of nucleotides shared between that species and the sperm whale. The positions of mammalian conserved sequence blocks 1 and 2 are indicated.

Figure 3.3

Alternative alignment of the mitochondrial control region sequence from cetaceans and cow. Species are as listed in Fig. 3.1. The sequence shown is the light strand in a 5′ to 3′ direction between $tRNA^{Pro}$ and $tRNA^{Phe}$. Dashed lines indicate insertions/deletions. Conserved blocks A through M previously identified in the literature are indicated. Blocks a, b, and c are those identified in this chapter.

Fig. 3.3

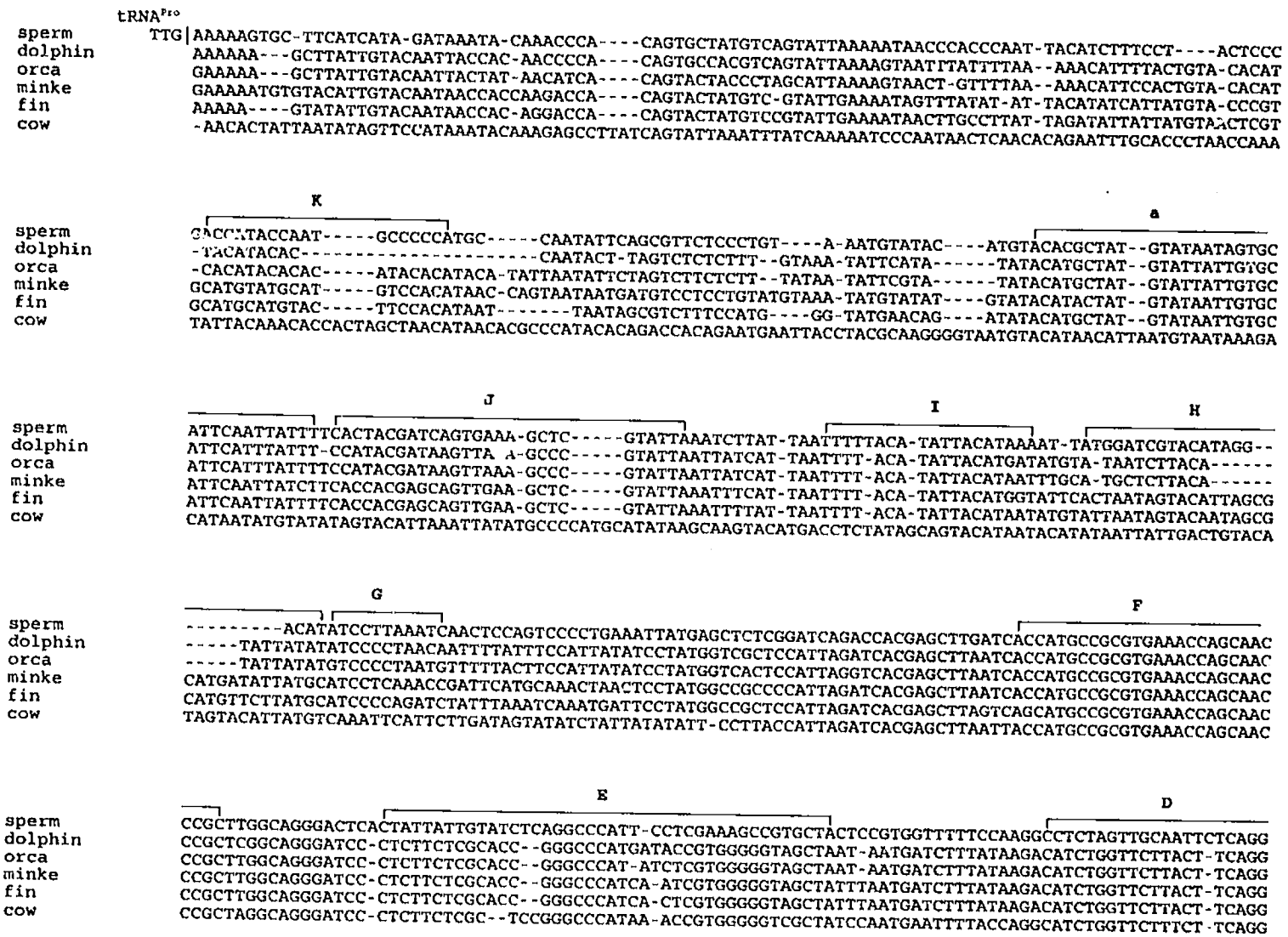

tRNA^Pro
sperm TTG|AAAAAGTGC-TTCATCATA-GATAAATA-CAAACCCA----CAGTGCTATGTCAGTATTAAAAATAACCCACCCAAT-TACATCTTTCCT----ACTCCC
dolphin AAAAAA---GCTTATTGTACAATTACCAC-AACCCCA----CAGTGCCACGTCAGTATTAAAAGTAATTTATTTTAA--AAACATTTTACTGTA-CACAT
orca GAAAAA---GCTTATTGTACAATTACTAT-AACATCA----CAGTACTACCCTAGCATTAAAAGTAACT-GTTTTAA--AAACATTCCACTGTA-CACAT
minke GAAAAATGTGTACATTGTACAATAACCACCAAGACCA----CAGTACTATGTC-GTATTGAAAATAGTTTATAT-AT-TACATATCATTATGTA-CCCGT
fin AAAAA----GTATATTGTACAATAACCAC-AGGACCA----CAGTACTATGTCCGTATTGAAAATAACTTGCCTTAT-TAGATATTATTATGTAACTCGT
cow -AACACTATTAATATAGTTCCATAAATACAAAGAGCCTTATCAGTATTAAATTTATCAAAAATCCCAATAACTCAACACAGAATTTGCACCCTAACCAAA
K
a
sperm GACCATACCAAT-----GCCCCCATGC-----CAATATTCAGCGTTCTCCCTGT----A-AATGTATAC----ATGTACACGCTAT--GTATAATAGTGC
dolphin -TACATACAC-----------------------CAATACT-TAGTCTCTCTTT--GTAAA-TATTCATA------TATACATGCTAT--GTATTATTGTGC
orca -CACATACACAC-----ATACACATACA-TATTAATATTCTAGTCTTCTCTT--TATAA-TATTCGTA------TATACATGCTAT--GTATTATTGTGC
minke GCATGTATGCAT-----GTCCACATAAC-CAGTAATAATGATGTCCTCCTGTATGTAAA-TATGTATAT----GTATACATACTAT--GTATAATTGTGC
fin GCATGCATGTAC-----TTCCACATAAT-------TAATAGCGTCTTTCCATG----GG-TATGAACAG----ATATACATGCTAT--GTATAATTGTGC
cow TATTACAAACACCACTAGCTAACATAACACGCCCATACACAGACCACAGAATGAATTACCTACGCAAGGGGTAATGTACATAACATTAATGTAATAAAGA
J
I
H
sperm ATTCAATTATTTTCACTACGATCAGTGAAA-GCTC-----GTATTAAATCTTAT-TAATTTTTACA-TATTACATAAAAT-TATGGATCGTACATAGG--
dolphin ATTCATTTATTT-CCATACGATAAGTTA A-GCCC-----GTATTAATTATCAT-TAATTTT-ACA-TATTACATGATATGTA-TAATCTTACA------
orca ATTCATTTATTTTCCATACGATAAGTTAAA-GCCC-----GTATTAATTATCAT-TAATTTT-ACA-TATTACATAATTTGCA-TGCTCTTACA------
minke ATTCAATTATCTTCACCACGAGCAGTTGAA-GCTC-----GTATTAAATTTCAT-TAATTTT-ACA-TATTACATGGTATTCACTAATAGTACATTAGCG
fin ATTCAATTATTTTCACCACGAGCAGTTGAA-GCTC-----GTATTAAATTTTAT-TAATTTT-ACA-TATTACATAATATGTATTAATAGTACAATAGCG
cow CATAATATGTATATAGTACATTAAATTATATGCCCCATGCATATAAGCAAGTACATGACCTCTATAGCAGTACATAATACATATAATTATTGACTGTACA
G
F
sperm ---------ACATATCCTTAAATCAACTCCAGTCCCCTGAAATTATGAGCTCTCGGATCAGACCACGAGCTTGATCACCATGCCGCGTGAAACCAGCAAC
dolphin -----TATTATATATCCCCTAACAATTTTATTTCCATTATATCCTATGGTCGCTCCATTAGATCACGAGCTTAATCACCATGCCGCGTGAAACCAGCAAC
orca -----TATTATATGTCCCCTAATGTTTTTACTTCCATTATATCCTATGGTCACTCCATTAGGTCACGAGCTTAATCACCATGCCGCGTGAAACCAGCAAC
minke CATGATATTATGCATCCTCAAACCGATTCATGCAAACTAACTCCTATGGCCGCCCCATTAGATCACGAGCTTAATCACCATGCCGCGTGAAACCAGCAAC
fin CATGTTCTTATGCATCCCCAGATCTATTTAAATCAAATGATTCCTATGGCCGCTCCATTAGATCACGAGCTTAGTCAGCATGCCGCGTGAAACCAGCAAC
cow TAGTACATTATGTCAAATTCATTCTTGATAGTATATCTATTATATATT-CCTTACCATTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCAAC
E
D
sperm CCGCTTGGCAGGGACTCACTATTATTGTATCTCAGGCCCATT-CCTCGAAAGCCGTGCTACTCCGTGGTTTTTCCAAGGCCTCTAGTTGCAATTCTCAGG
dolphin CCGCTCGGCAGGGATCC-CTCTTCTCGCACC--GGGCCCATGATACCGTGGGGGTAGCTAAT-AATGATCTTTATAAGACATCTGGTTCTTACT-TCAGG
orca CCGCTTGGCAGGGATCC-CTCTTCTCGCACC--GGGCCCAT-ATCTCGTGGGGGTAGCTAAT-AATGATCTTTATAAGACATCTGGTTCTTACT-TCAGG
minke CCGCTTGGCAGGGATCC-CTCTTCTCGCACC--GGGCCCATCA-ATCGTGGGGGTAGCTATTTAATGATCTTTATAAGACATCTGGTTCTTACT-TCAGG
fin CCGCTTGGCAGGGATCC-CTCTTCTCGCACC--GGGCCCATCA-CTCGTGGGGGTAGCTATTTAATGATCTTTATAAGACATCTGGTTCTTACT-TCAGG
cow CCGCTAGGCAGGGATCC-CTCTTCTCGC--TCCGGGCCCATAA-ACCGTGGGGGTCGCTATCCAATGAATTTTACCAGGCATCTGGTTCTTTCT-TCAGG

Fig. 3.3 (continued)

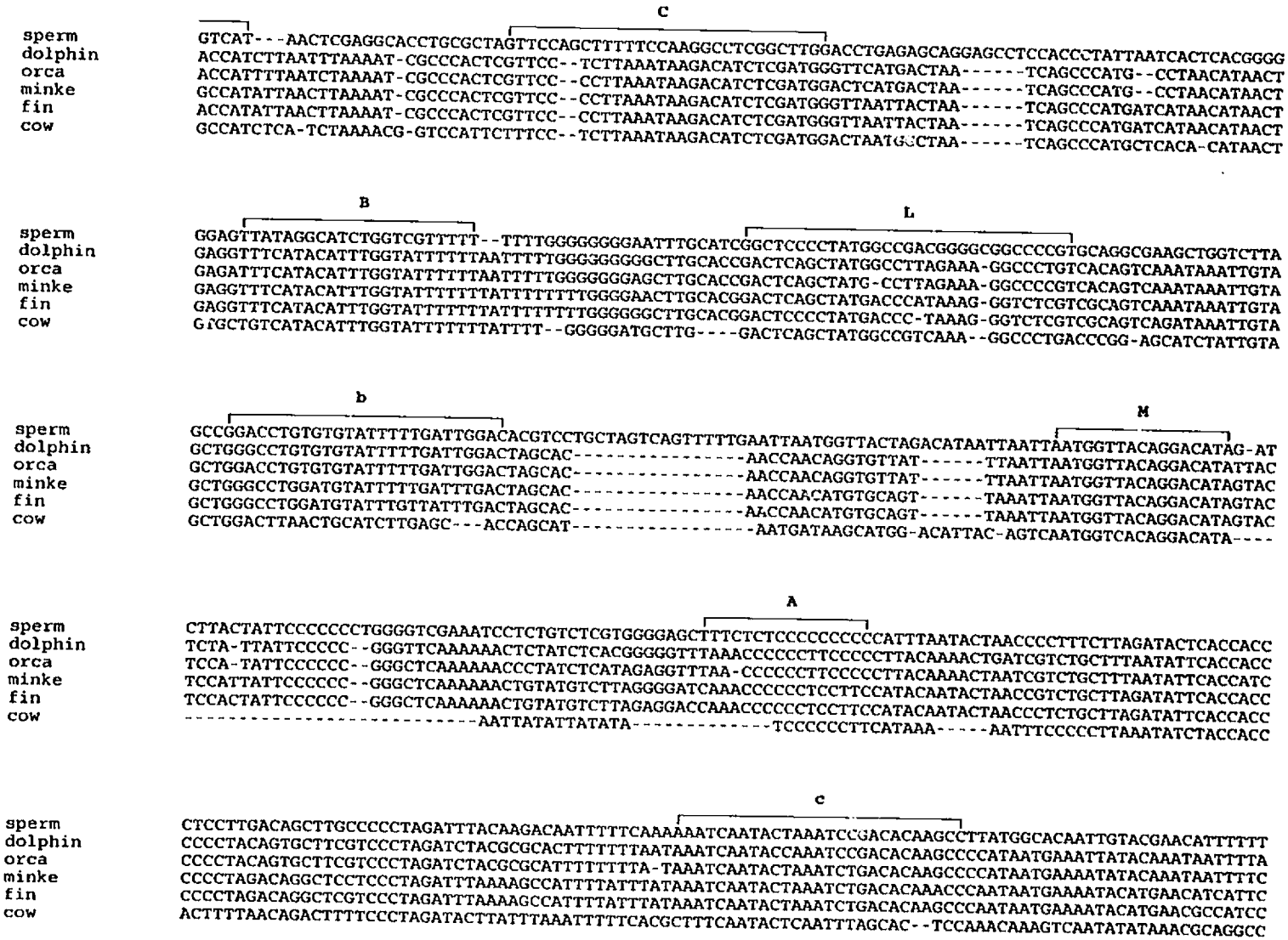


Fig. 3.3 (continued)

```
                                  tRNA^Phe
sperm           CG-TATATAATTGG|GTT
dolphin         TACTCCACAA----
orca            TGCCCCCCTCCCCC
minke           CTATTTACA--TAC
fin             CTATCCA-A--TAC
cow             CCCCCC----CCCC
```

and E are highly conserved between the four mammals. This is not the case for the sperm whale sequence as it differs markedly in these regions, having only 36 to 63% similarity with the other cetaceans (Fig. 3.2).

Blocks L and M have been found only in cetacean and bovine sequences (Anderson *et al.*, 1982; Southern *et al.*, 1988; Hoelzel and Dover, 1991). Block M is well conserved between the six species here, with 100% sequence identity among the cetaceans, and with 94% sequence identity between sperm whale and cow. Block L, however, is much less conserved between sperm whale and other cetaceans with only 59-65% sequence identity. Three blocks were identified in this study that have not been previously described. One is at the 5′ end of the control region (Block a, Figures 3.1 and 3.2) while the other two are 3′ to the central region (Blocks b and c). Among the cetaceans, these regions share 77-100% sequence identity with the sperm whale.

In earlier studies, discussion of conserved elements focused on three CSBs (CSB-1, CSB-2, and CSB-3) identified by Walberg and Clayton (1981). It has been reported that CSB-3 is not present in the bovine control region (Anderson *et al.*, 1982; Brown, 1985; Foran *et al.*, 1988) and it seems also to be absent in the cetacean control region. CSB-1 corresponds to block M, while CSB-2 is at approximately the same position as block A (Figures 3.1 and 3.2), except in the case of the bovine sequence where CSB-2 is located immediately adjacent to

$tRNA^{Phe}$ (Anderson *et al.*, 1982; Low *et al.*, 1988).

The presence of conserved blocks in the control region of many species (Walberg and Clayton, 1981; Anderson *et al.*, 1982; Brown *et al.*, 1986; Foran *et al.*, 1988; Southern *et al.*, 1988; Bogenhagen and Morvillo, 1990; Hoelzel *et al.*, 1991) has led to speculations about their function in transcription and replication of the mtDNA. These include a role for CSBs in switching from RNA to DNA synthesis in mtDNA (Chang and Clayton, 1985), relief of supercoiling during mtDNA heavy strand synthesis (Low *et al.*, 1987), and substrate recognition by a mitochondrial RNA processing enzyme (Bennett and Clayton, 1990).

While CSBs may have important roles in certain species, there are many examples where these elements are not well conserved, and therefore, alternative explanations are required. In an alignment of four rat and one mouse species, Brown *et al.* (1986) found that CSB-1 had many insertions/deletions, and suggested that at most, only part of this sequence is necessary for mtDNA heavy strand synthesis. In an alignment of four hominids, substitutions were found in all three CSBs and part of CSB-2 and 35% of CSB-3 were deleted in the gorilla (Foran *et al.*, 1988). In a broader phylogenetic analysis of the control region, Bogenhagen and Morvillo (1990) found that CSB-1 is not well conserved between *Xenopus*, mouse, rat and human.

Block M, which is well conserved among the cetaceans and

the bovine sequence, corresponds to CSB-1. Also, block A is similar to CSB-2. Although the sequence conservation of block A is not high between the sperm whale and the other cetaceans, a tract of cytosine residues is a common feature. Low *et al.* (1988) reported that specific endonucleases that may function in mtDNA heavy strand synthesis have specificity for this tract, despite the fact that this block is found in different locations in different species (*e.g.* the bovine sequence). It appears, therefore, that certain nucleotides in a CSB may be essential while others are not. It may also be possible that in some cases the exact nucleotide sequence can change while the necessary protein-DNA interactions still function. Fisher *et al.* (1989) reported that between the human and mouse binding site for mitochondrial transcription factor 1, only 7 of 22 nucleotides are identical, yet, the transcription factor from either species can locate and bind to the promoter of the other. This indicates that there may be substantial flexibility in binding site recognition by mitochondrial transcriptional factors.

This study has shown that many of the blocks previously identified in cetaceans (Southern *et al.*, 1988; Hoelzel and Dover, 1991) are not conserved in the sperm whale. This lack of conservation among cetaceans of certain blocks suggests that these elements are not essential for the functioning of mtDNA. Two blocks that may be important, however, are the well-conserved sequences that correspond to CSB-1 and CSB-2,

which have been identified in a wide range of animals.

In terms of more fully understanding the roles of conserved sequence elements, it may be important that, among cetaceans, the sperm whale's evolutionary position is uncertain. In this study the sperm whale was compared to orca and dolphin, members of the family Delphinadae, and fin and minke whales, members of the family Balaenopteridae. While the work in this chapter does not help resolve the sperm whale's uncertain position, it does reaffirm this species' unique position among cetaceans. A greater appreciation of sequences in the control region important for the functioning of the cetacean mtDNA might be derived from an analysis of species more closely related to the sperm whale than those used here, such as the dwarf and pygmy sperm whales (members of the same family as the sperm whale, Physteridae), as well as members of the Ziphiidae (beaked whales) which have not been well represented in such studies.

Summary

While some CSBs in the cetacean control region were also found to be conserved in the sperm whale, others were not, calling into question the role of these CSBs in the proper functioning of the mtDNA genome. Three new cetacean CSBs were identified in this analysis.

CHAPTER 4: Analysis of social organization in groups of female and immature sperm whales using mitochondrial DNA and microsatellite loci

Introduction

Sperm whales have had a long history of interactions with humans, having been hunted extensively during the "Yankee" whaling period of 1712-1925, and more recently in the modern whaling era (1950's to 1980's) (*e.g.* Rice, 1989). Thus, whalers amassed considerable information on the natural history of sperm whales. This information was somewhat limited, however, being collected usually at one moment in the lives of the whales: that of unnatural death by harpoon. Despite the limitations of these data, observations by whalers provided the first insights into the unusual social organization of groups of female and immature sperm whales.

Caldwell and Caldwell (1966) and Caldwell *et al.* (1966) reviewed the whaling literature and reported numerous instances of adults caring for young, and adults caring for injured companions, both adults and calves. Some whalers speculated that aunts help in the rearing of calves. Oshumi (1971) also proposed a matrilineal organization in sperm whale groups and reported that some females marked in the same social group were recovered as much as 10 years later. Marking in this study was carried out using stainless steel "Discovery" tags, which were embedded into the blubber of the

whale and recovered when the whale was killed and processed. More recently, non-invasive research spanning several years has suggested that social groups actually contain two or more "units", where units are the entities that stay together over periods of years (Whitehead *et al.*, 1991). Again, there is speculation that units are made up of family members with a matrilineal organization.

While several lines of evidence suggested female-centred family groups, genetic testing was needed to confirm this hypothesis. In an effort to address this genetically, Richard (1995) used nuclear DNA markers (microsatellite loci) to evaluate the possibility of kin selection in groups of female and immature sperm whales. Individuals from 3 well-defined groups off Ecuador, as well as presumably unrelated individuals not from these social groups, were assayed with 5 microsatellite loci, and were sexed using molecular methods. The resulting allele frequencies were tested against 5 models of group composition: (1) 1 matriline; (2) 2 matrilines; (3) unrelated mothers, each accompanied by 2 offspring; (4) half-sibs; and (5) unrelated individuals. Models 1-3 were thought to represent possible associations in sperm whales, while 4 and 5 were to represent extreme values of relatedness and unrelatedness, respectively. The microsatellite analysis found that allele frequencies in observed sperm whale groups were consistent with models of 1 or 2 matrilines, but not with any other model tested. In addition, this work showed that

groups consist primarily of females (79% overall), and estimated that males leave their family groups at around 6 years of age.

Richard's (1995) data provided the first genetic evidence that sperm whale groups were composed of related females and their offspring, but also indicated that further genetic analyses were necessary. In particular, the suggestion of matrilineal group structure is best tested using a maternally-inherited marker. Therefore, I conducted mitochondrial analyses on individuals in the same 3 groups. The work presented in this chapter represents a unique combination of extensive field observations, nuclear data and molecular sexing (K. Richard), and mitochondrial DNA information (M. Dillon) to examine social organization in groups of sperm whales more fully than has been possible previously.

Materials and methods

Field work was conducted near mainland Ecuador (1°N - 3°S, 81°W-82°W, from January to March, 1991. Working from a sail boat, sperm whales were tracked acoustically day and night using hydrophones and visually during daylight hours. Groups of whales were followed for variable periods of time, from a few hours to a few days. While with groups of whales, we took fluke photographs for individual identifications (Arnbom, 1987) from as many different whales as possible, and we searched for sloughed skin in the wake of swimming whales and in the slicks the whales left behind when they started

deep feeding dives (Whitehead *et al.*, 1990; Amos *et al.*, 1992). Skin samples were handled as described elsewhere (sec. 2.1). Some samples (approximately 20%) were linked to fluke identification photographs, and therefore were from known individuals, while the majority of samples were from unidentified individuals.

4.1 Definition of social groups

As described by Richard (1995), three social groups were identified for this analysis. These groups consisted of whales travelling together and interacting over periods of hours to days. Two groups, A and B, were each considered to be discrete in that they did not appear to mix with other social groups over the periods of observation. These groups were each followed for 2 consecutive days and identification photographs were taken, with many identifications being common to both days. Data from group C were collected during an intense, highly interactive gathering of 20-30 whales, which lasted about one hour. Although many skin samples were collected from this group, it was not possible to take fluke identification photographs. The interaction among member of group C seemed highly social, but the social bonds in this group are less well defined than for groups A and B, for which we have more observational data.

Adult males, which are easily identified in the field due to their distinctive size, were not observed in any of the groups during the periods in which samples were collected.

4.2 Molecular sexing

Except for the mature males, it is extremely difficult to determine the sex of living sperm whales at sea. Therefore, sex was determined using molecular methods, whereby the male-specific *SRY* gene was PCR amplified (Richard *et al.*, 1994); results were confirmed using an X-linked microsatellite locus (Richard *et al.*, 1996).

4.3 Mitochondrial analysis

The sperm whale mitochondrial control region was amplified and directly sequenced using the exonuclease I / shrimp Alkaline Phosphatase digestion method described by Werle *et al.* (1994). Five μL of PCR product were incubated at 37°C for 1 hour with 10 units Exonuclease I and 2 units of shrimp alkaline phosphatase (United States Biochemical). Enzymes were inactivated by heating to 80°C for 15 min. Volume was adjusted to 10 μL with ddH_2O; primers were annealed by adding 1 μL of 20μM sequencing primer, 2μL annealing buffer and 1 μL DMSO. The mixture was heated to 100°C in a thermal cycler for 3 min. and immediately cooled on ice. Sequencing primers were $tRNA^{Thr}$ (one of the PCR primers) and an internal primer that annealed to "block a" of the sperm whale control region (chapter 3). With these primers, 500-600 bp of the sperm whale control region were sequenced. Sequences were aligned and variable nucleotide positions identified. Based on the sequence at these positions, mtDNA haplotypes were defined. I checked the accuracy of these sequences by

resequencing in excess of 8,000 bp, with no discrepancies observed.

4.4 Number of individuals in group with particular mtDNA haplotype

For a randomly chosen individual, the mean number of individuals within its group with its mtDNA haplotype was estimated from:

$$\sum_i n_i \cdot n_i \cdot (T / \sum_i n_i) / (\sum_i n_i)$$

where n_i is the number of animals in the group known to have mtDNA haplotype i, and T is the total number of animals in the group. T was estimated using mark-recapture methods (Seber, 1982) on medium to high quality individual identification photographs ($Q \geq 3$, Arnbom, 1987), and is known only for groups A and B for which we have good photographic identification records.

4.5 Microsatellite analysis

Multilocus genetic profiles were constructed for each sample using 5 microsatellite markers isolated from sperm whales, using methods presented elsewhere (Richard, 1995; Richard *et al.*, 1996).

Because there were more skin samples than the total number of whales, the first step in the microsatellite analysis was to determine which samples were repeated samples from the same whales. If two samples were identical at all

microsatellite loci, they were assumed to be from the same individual, and only one of these samples was used in the analysis of social structure (Richard, 1995).

4.6 Combining molecular data to assess kinship

Computer analyses and modelling to investigate kinship were performed by H. Whitehead. In this analysis, comparisons of the mean number of microsatellite alleles shared were made between all pairs of individuals in the same group with a particular mtDNA haplotype and those in the same group with a different haplotype. Similar comparisons were made for pairs of whales in different groups. Significance was tested using jackknifing, in which each sample is omitted from the analysis in turn, and approximation to the t-distribution (Sokal and Rohlf, 1981).

The observed genetic results were tested against a model of perfect matrilines, where males disperse (estimated to be around age 6 years, Richard, 1995) but females do not. The expected mean number of parent-offspring relationships per individual in a matrilineal group was compared to the estimated number. This estimation was carried out as follows. For a pair of individuals to be considered potential parent-offspring, they had to share at least one microsatellite allele at each locus (we are assuming Mendelian, co-dominant inheritance of microsatellite alleles) and have the same mtDNA haplotype. These potential parent-offspring relationships could be real, or they could be artificial, where allele

sharing in non-parent-offspring was due to identity by state, but not by descent. Such artificial parent-offspring pairs might be expected to be more common when fewer alleles are examined, and when the alleles are not highly variable. In addition, more non-parent-offspring pairs might be seen in family groups, where, for example, an aunt and nephew or cousins might appear as parent-offspring. The expected number of artificial parent-offspring pairs was estimated from the frequency distribution of microsatellite alleles and the number of individuals with different mtDNA haplotypes in each group. Then, for each group, the true number of parent-offspring relationships among the typed individuals was estimated by subtracting the number of artificial-parent-offspring pairs from the observed number. From this, the average number of parent-offspring relationships per individual was calculated by dividing by the number of typed individuals in the group and then multiplying by the ratio of the estimated group size to the number of typed individuals within the group. For group C no population estimate was available, so that only a lower bound for this measure could be calculated.

These steps were carried out separately on each group for the number of female parents-offspring of a female, $O(♀,♀)$, the male offspring of a female, $O(♀,♂)$ and the female parents of males, $O(♂,♀)$. Expected numbers were calculated assuming the population parameters of sperm whales used by the

International Whaling Commission (1982), and a population at equilibrium:

$$Ex(O(♀,♀)) = \sum_{a=1}^{\infty} (1-ß)^a \left[(1-ß)^a + \sum_{b=f}^{a-1} p.\sigma.(1-ß)^{a-b-1}/2 \right] / \sum_{a=1}^{\infty} (1-ß)^a$$

$$Ex(O(♀,♂)) = \sum_{a=1}^{\infty} (1-ß)^a \left[\sum_{b=\max(f,a-d)}^{a-1} p.\sigma.(1-\alpha)^{a-b-1}/2 \right] / \sum_{a=1}^{\infty} (1-ß)^a$$

$$Ex(O(♂,♀)) = \sum_{a=1}^{d} (1-\alpha)^a \cdot (1-ß)^a / \sum_{a=1}^{d} (1-ß)^a$$

where α is the mortality of males over age 1 (0.066/yr), ß the mortality of females over age 1 (0.055/yr), σ the mortality between birth and age 1 (0.093/yr), p the birth rate of mature females (0.20/yr), f the age of first birth for females (10 years), and d the age of dispersal for males (6 years, see below). In this analysis, it is also assumed that genetic samples were only obtained from animals 1 year of age and older (consistent with our observations in the field).

Results

4.7 Relatedness of individuals within groups

Groups consisted primarily of females (Table 4.1). The overall group structure suggested that these females are related, as each group was dominated by one mtDNA haplotype, although all groups contained more than 1 haplotype (Fig. 4.1). Additionally, microsatellite allele sharing showed that

Table 4.1. Data collected, estimated numbers, sex ratio, and distribution of mtDNA haplotypes in 3 groups of sperm whales.

	Group A	Group B	Group C
ID photographs taken	87	113	0
Estimated numbers (SE)*	28.1 (1.5)	24.3 (0.8)	-
Skin samples	48	56	33
Genetically identified individuals	18 (17)+	20 (18)+	18(15)+
Sex ratio	12♀,6♂	16♀,4♂	16♀,2♂
mtDNA haplotypes:			
#1	1♀,2♂	1♀	7♀,1♂
#2	1♂	14♀,3♂	3♀
#3	9♀,3♂	1♀,1♂	
#6			1♀
#10			3♀
#12	1♀		2♀,1♂
Unable to type	1♀		

* Population estimates calculated using unbiased Petersen mark-recapture methods (Seber, 1982) on photographs of moderate to high quality: Arnbom's $Q \geq 3$ (Arnbom, 1987).
\+ Numbers of individuals typed at all 5 microsatellite loci are given in parentheses.

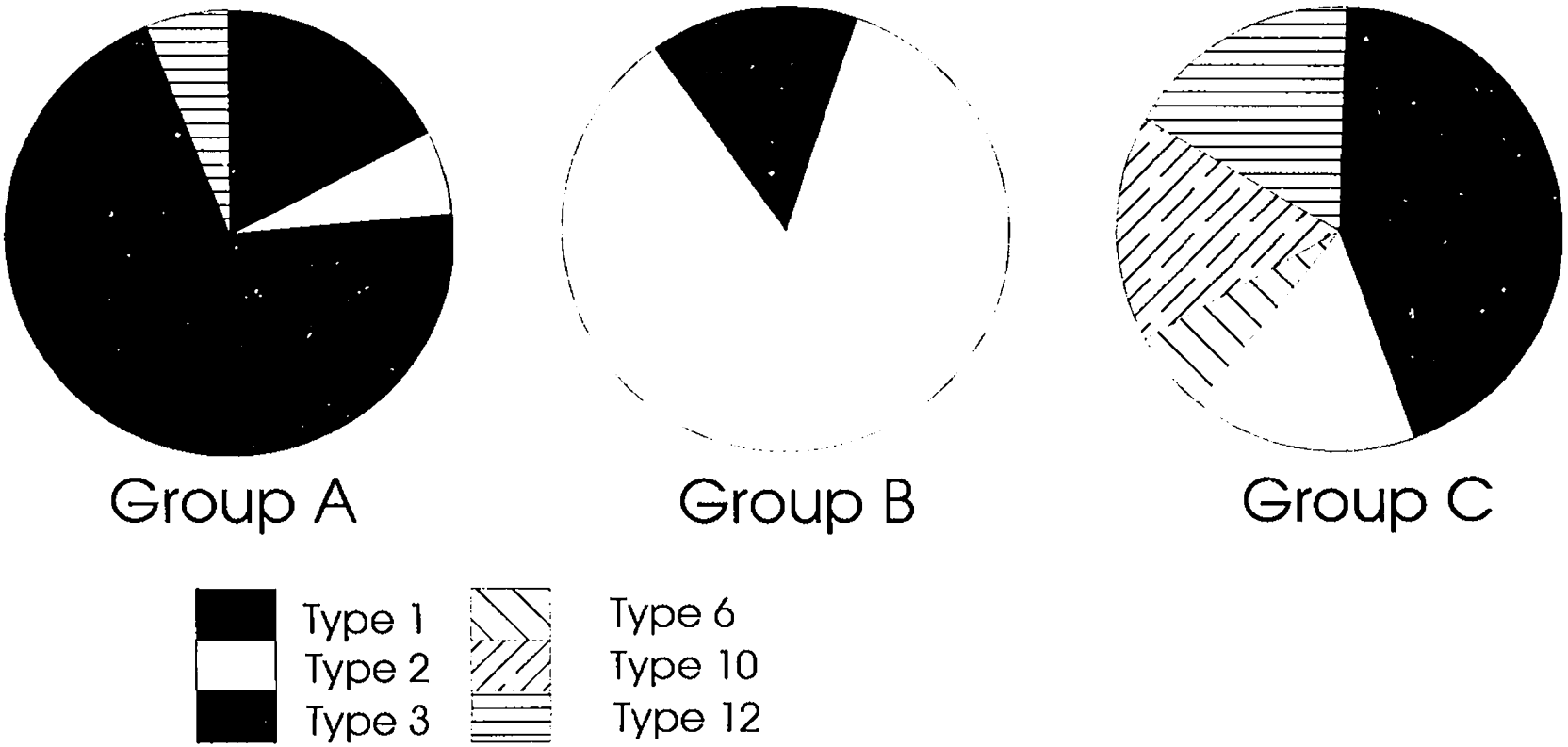


Figure 4.1: Frequencies of mitochondrial haplotypes in 3 sperm whale groups off mainland Ecuador.

pairs of individuals in the same group had significantly more alleles in common than pairs of whales in different groups (Table 4.2).

4.8 Genetic structure of groups

Within groups, there was more similarity of microsatellite alleles between pairs of individuals with the same mtDNA haplotype than between those with different mtDNA haplotypes, but the difference was small and not significant (Table 4.2).

Two of the three groups, A and B, had fewer estimated parent-offspring relationships per individual than expected from perfect matrilines (Table 4.3). Group C fits the model more closely. A striking deviation from the model was that in group A, there was a lack of potential mothers of the males, where only 1 of 6 males in the group had a potential mother among the typed females.

We also found more similarity at microsatellite loci among individuals in the same group but with different mtDNA haplotypes than among individuals in different groups, and the difference was significant ($p=0.05$, Table 4.2). A possible explanation for this is that individuals within groups but with different mtDNA haplotypes may be related through their fathers, not their mothers.

Table 4.2. Mean number of shared alleles for individuals in the same and different groups, sharing and not sharing mtDNA haplotypes.

	Mean shared alleles*	Tests+
Within groups, within haplotypes	3.17	
Within groups, between haplotypes	3.10	$P=.4$
Between groups, within haplotypes	3.00	$P<.001$ $P=.05$
Between groups, between haplotypes	2.83	$P=.2$

* Mean number of shared alleles (out of 9) at 5 microsatellite loci (including one x-linked locus at which a maximum of only one shared allele was counted).

\+ One-tailed significance tests for differences between means used the Jackknife procedure and t-distribution approximation (49 d.f.).

Table 4.3. Estimated mean number of parent-offspring relationships per individual from mtDNA and microsatellite data, compared with expected mean numbers of true parent-offspring relationships per individual in matrilineal groups.

	Estimated mean number of parent-offspring relationships of 1 female with		of 1 male with
	females	males	females
Group A	0.55	-0.02	-0.03
Group B	0.20	0.12	0.79
Group C	>0.74	>0.17	>1.36
Expected	0.94	0.23	0.83
Expected ß=0.11*	0.61	0.10	0.69

* Expected numbers if the natural mortality of females is doubled to ß=0.11/yr.

Discussion

The results presented in this chapter represent the first comprehensive study of the genetic basis for social organization in groups of sperm whales using a combination of nuclear and mitochondrial markers. This analysis revealed that social groups consist mainly of related females and their offspring. This social structure, in which related females appear to stay together for periods of at least years, would provide suitable conditions for the evolution and maintenance of communal care of calves, which has long been observed in this species. Given that all groups contained more than one haplotype, the results are in agreement with group structure proposed by long-term photoidentification studies of sperm whales (Whitehead *et al.*, 1991), which suggested that units are family groups that stay together over time; units temporarily merge, probably to facilitate efficient foraging.

Some results of this analysis were, however, unexpected. For example, it is difficult to resolve the problem of the lack of parent-offspring relationships in groups A and B. Group A is especially interesting in this regard, as this group had the highest proportion of males. By virtue of the fact that the males are small, we know they are young (probably less than 6 years; Richard, 1995); thus we know they are offspring and not parents and we can look for mothers of these young males among the typed individuals. This is in contrast to most of the parent-offspring relationships between

2 females examined in this study, where the ages of the individuals are not known, and therefore, we do not know which individuals are the parents and which are the offspring. In group A, 5 of the 6 males did not have potential mothers among the typed whales. Several explanations for this are possible including: (1) the mothers are among the 10 whales estimated to be missed in the genetic sampling (Table 4.1). This requires that the non-sampled whales included *all* 5 missing mothers, despite the fact that the offspring of these mothers (who might be expected to associate closely with their mothers) were all sampled. (2) There could be movement of females between groups. Although we cannot say with certainty that females have left this group, the observation of a single female in group A with haplotype 12 (Table 4.1) suggests that a female may have moved into this group, and provides evidence of movement of females from group to group. (3) There could be movement of young males between groups. One male in the group had a haplotype (type 2) not seen in the rest of group A, and would be most consistent with that male joining the group. (4) There could be errors in the assumed population parameters. For example, if the assumed mortality of adult females were doubled (not implausible given the substantial whaling that took place in nearby Peruvian waters as recently as 1983 (Ramirez, 1989)) the estimated and expected numbers of parent offspring relationships become closer (Table 4.3).

For group B, it is less likely that parents or offspring were missing from the sample, as 20 of an estimated 24 whales in the group were genetically sampled. One female in this group had a unique haplotype, suggesting movement of females, but there was no evidence of males moving into this group, as all males shared microsatellite alleles at all loci and mtDNA haplotypes with females in the group. Again, doubling the female mortality rate improved the discrepancy between expected and observed parent-offspring values.

It is not possible to determine which of these scenarios, if any, accounts for the patterns observed. Indeed, it may be a combination of events, where one group A mother was missed from the sample, one died prematurely, one left the group and so on. Further examination of social groups in detail may shed light on group structure and the movement of females among groups.

An additional, unexpected result of these genetic analyses was the suggestion that individuals within a social group may be related paternally. In cetaceans studied to date, there have been no reports of paternal leakage of the mitochondrial genome (Arnason *et al.*, 1991a; McMillan and Bermingham, 1996) and therefore we assume that the inheritance of mtDNA is strictly maternal in sperm whales also. Indications of paternal relatedness were surprising because Best (1979) and Whitehead (1993) have reported that males stay with social groups of females for brief periods only. In

addition, groups in the Galápagos seem to have just one calf per year (Whitehead, 1993). Thus it is difficult to explain paternally-related half-sibs. Other information, however, lends support to paternal relatedness. Receptive females may be encountered in the same group, as there is evidence that females within groups synchronize their oestrous periods (Best and Butterworth, 1980), and Whitehead (1993) observed males reassociating with the same group over a period of days. Also, Best (1979) reports that there exists "strong evidence for a single male parent for several fetuses in one harem school" based on colour phenotypes, but these data were not published, and it is impossible to assess the validity of the technique.

While the suggestion of paternal relatedness is intriguing, it is also indicative of our lack of knowledge of sperm whale mating in that some evidence supports such a finding while other data argue against it. Information on the frequency of visits by mature males to the breeding grounds, the extent to which males fight for dominance and access to females, the effect of removal of large males by whalers, and the role of female mate choice is needed to increase our knowledge of sperm whale reproduction.

More insight may be gained by further studies which combine observational and genetic data. In particular, long-term tracking of mature males (*e.g.* by satellite tagging) and the development of highly-polymorphic male-specific markers

would be informative.

Summary

Overall, the results presented in this chapter provide evidence for the relatedness of members of social groups of sperm whales, and provide insight into the evolution and maintenance of some of the social behaviours for which sperm whales are well known. By combining different genetic markers, we were able to gain more insight into genetic structure of these groups than would have been possible with one set of molecular markers alone.

CHAPTER 5: Genetic structure of populations of female and immature sperm whales assessed by mitochondrial DNA sequence variation over a range of social and geographic scales

Introduction

Despite considerable information gathered on sperm whales from the whaling industry and from non-invasive studies in the last decade (*e.g.* Whitehead and Gordon, 1986), gaps in our knowledge of this species remain. In particular, although it is known that groups of females are remarkably social (Caldwell and Caldwell, 1966), the basis for this sociality is not fully understood. In addition, the patterns of movement of sperm whales within and among oceans are not known. A better understanding of these aspects of sperm whale biology is central to assessing the effects of past whaling and making conservation decisions in the future.

As reviewed in chapter 1, dimorphism between male and female sperm whales is extreme, with differences in physical attributes, geographic distribution and social structure. While males form loose aggregations or are found singly, observations of groups of female and immature sperm whales reveal tight social bonds. Social behaviours seen in groups of female and immature sperm whales include remaining with injured companions (Caldwell *et al.*, 1966; Caldwell and Caldwell, 1966) and communally caring for calves (Arnbom *et al.*, 1987; Gordon, 1987; Arnbom and Whitehead, 1989; and

Whitehead 1996).

There is evidence both from whaling (Oshumi, 1971) and photographic studies (Whitehead *et al.*, 1991) that some female sperm whales stay together for periods of years. This led to suggestions that groups consisted of related females, and that sociality was maintained by kin selection, as opposed to some form of reciprocity or mutualism between unrelated individuals. This was merely conjecture until genetic studies could be applied to sperm whales. Now, the genetic structure of such groups is beginning to be elucidated. In chapter 4, I presented results of microsatellite and mitochondrial analysis that showed that members of 3 groups of female and immature sperm whales off mainland Ecuador were related, and that group structure was consistent with one or more matriline.

The extent of movement of such groups of female sperm whales within and among oceans is not well known. Tagging studies (Oshumi, 1971; Ivashin, 1977, 1981; Kasuya and Miyashita, 1988) conducted in conjunction with the whaling industry had the potential to provide valuable information on the movements of female sperm whales, but due to low recovery rates (only 3-4% of tagged sperm whales were recovered) and the bias towards large males, these studies have limited applicability to females. For example, in one study of 45 sperm whales marked and recovered between 1952 and 1979, only 5 were females; 4 of the females were recovered 3 years or less after being tagged (Ivashin, 1981). More recently,

photoidentification techniques have been developed for sperm whales as an alternative to invasive approaches (Arnbom, 1987). With these methods many whales can be identified in a short time, without the biases of the whaling industry. Dufault and Whitehead (1995) used this approach to show that mature female sperm whales have limited ranges and make movements in the order of 1000 km or less. Again, however, the time scale is limited, with the maximum time between resightings being less than 10 years.

An understanding of the movements of female sperm whales is essential to the definition of stocks, upon which management and conservation decisions should be based. However, only a few studies on sperm whale stocks have been carried out, and many of the results are conflicting (reviewed by Best, 1974; Donovan, 1991). Given the importance of having accurate knowledge of sperm whale stocks, it is clear that further investigations are required.

In this study, I used mtDNA control region sequences to (1) examine the amount of genetic variability in sperm whales; (2) estimate the genetic relatedness of members of social groups of female and immature sperm whales; and (3) evaluate the extent of movement of female sperm whales within the South Pacific and among the Pacific, Atlantic and Indian Oceans, over larger time scales than have been investigated previously.

Materials and methods

5.1 Field work

Tissue samples were obtained by following sperm whales from a sailboat and using a dipnet to collect skin naturally sloughed from the whales (Whitehead *et al.*, 1990; Amos *et al.*, 1992). Some samples were collected when only one whale was in sight. If a fluke identification photograph was also taken (Arnbom, 1987), the skin was assumed to be from the identified individual, as we have observed that sloughed skin sinks quickly (Whitehead *et al.*, 1990). Other samples were from groups of several whales, and were not linked to identification photos; in these cases there is the possibility of repeated samples from the same whale. This was handled as described in sec. 5.4 ii.

At sea, samples were preserved in a saturated NaCl solution, or in saturated NaCl with 20% dimethylsulfoxide (DMSO), following Amos and Hoelzel (1991). Upon return to the laboratory, samples were stored at -20°C.

5.2 DNA extraction, PCR amplification and sequencing

DNA was extracted from skin samples using organic extraction (Amos and Hoelzel, 1991) or by boiling in a solution containing a chelating resin ("Chelex" or "Instagene", BioRad; Walsh *et al.*, 1991) and using this as a template for amplification.

PCR amplification, exoI/sAP purification and direct sequencing were done exactly as described in sec. 4.3, where

the entire control region was amplified using cetacean-specific primers, and the sequencing primers were $tRNA^{Thr}$, and an internal primer that annealed to "block a" of the sperm whale control region (chapter 3). The sequence from these primers overlapped and gave 500-600 bp at the 5' end of the control region.

The accuracy of the mtDNA sequencing was confirmed by resequencing more than 13,500 bp (*i.e.* an additional 5,500 bp over what was done in sec. 4.3) with no discrepancies observed. This included samples that were sequenced four times. Among those resequenced were skin samples collected on different days, but believed to be from the same individual based on fluke identifications. In every case, the haplotypes were identical, demonstrating the reliability of assigning skin samples to identified whales.

5.3 Sequence alignment and identification of haplotypes

Sperm whale control region sequences were aligned using CLUSTAL V (Higgins and Sharp, 1988). Based on the sequence at variable nucleotide positions, distinct haplotypes were defined.

5.4 Analysis of haplotypes

i. *Geographic location of samples*

Samples from 9 locations worldwide (Fig. 5.1) were hierarchically categorized according to the following 5 levels: (1) Oceans (Pacific, Atlantic or Indian); (2) Areas within oceans. Areas were geographically separate (several

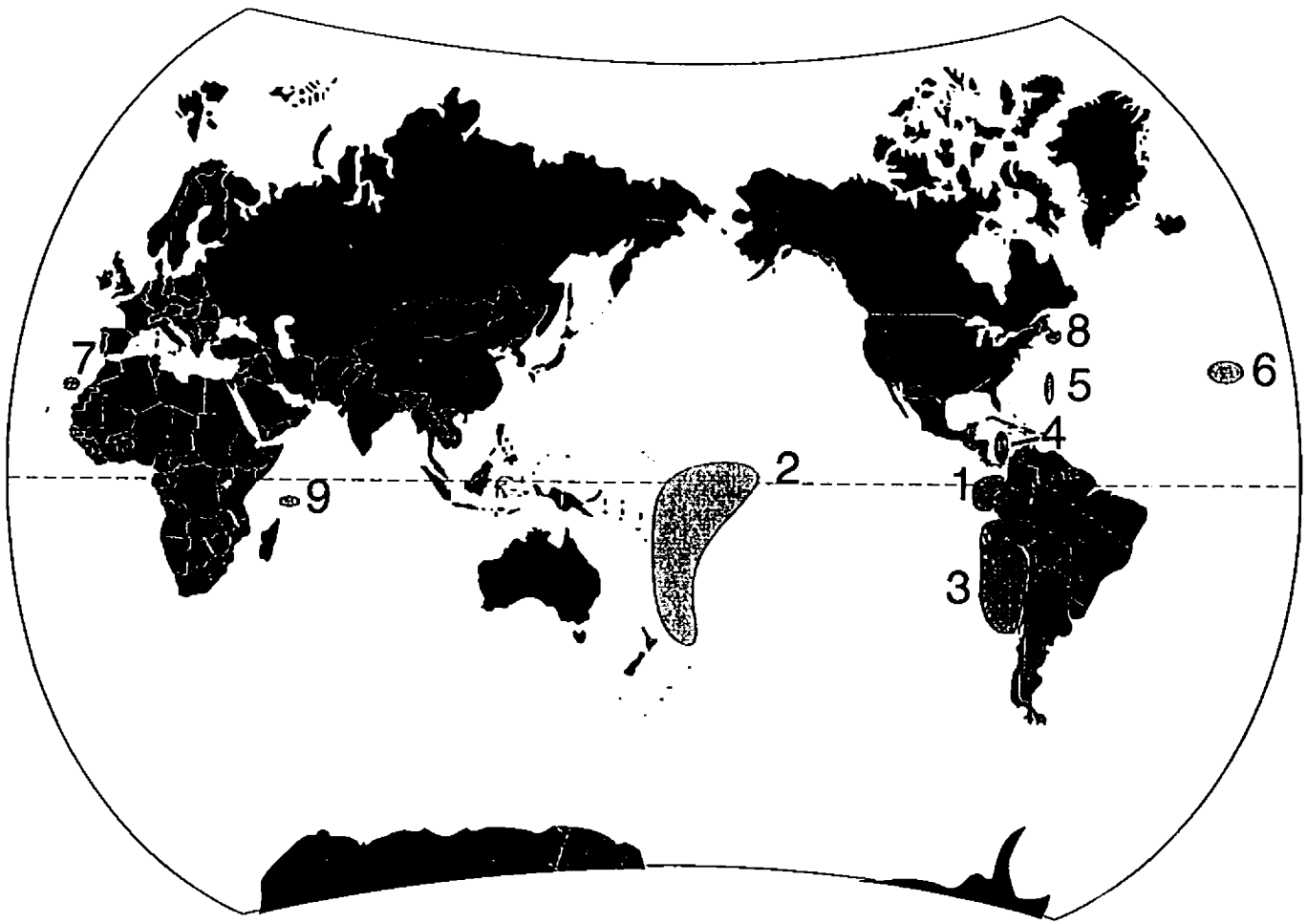


Figure 5.1: Geographic location of sperm whale samples, and sample sizes in each area. Shaded regions represent separate sampling areas which were: 1 - Galapagos, Ecuador, N. Peru (N=92); 2 - W. South Pacific (N=26); 3 - E. Pacific south of 10° S (N=22); 4 - Caribbean (N=10); 5 - S.W. North Atlantic (N=6); 6 - Azores (N=3); 7 - Canary Islands (N=3); 8 - Nova Scotia (N=1); 9 - Seychelles (N=19).

thousand km apart) regions within an ocean. In the South Pacific, Ecuador/Galápagos/ Northern Peru, Western South Pacific and Eastern South Pacific south of 10°S were considered areas. In the Atlantic Ocean, the Azores, Canary Islands, Nova Scotia, Caribbean, and SW N. Atlantic were classified as separate areas. Finally, the Seychelles samples from the Indian Ocean were classified into one area; (3) Places within areas. Areas were more finely divided into places (ca. 1000 km apart); for example, Ecuador and the Galápagos Islands were considered separate places within an area; (4) Groups were defined as whales within a place which co-ordinated their behaviour, interacting socially, showing synchrony of heading, etc. (5) Individuals were identified by unique patterns on the trailing edge of their tailflukes (Arnbom, 1987).

ii. *Analysis programs*

a. *AMOVA*

I used the Analysis of Molecular Variance program (AMOVA v. 1.55; Excoffier *et al.*, 1992) to examine mtDNA variation at different geographic levels (oceans, areas, places and social groups). The program performs, for any 2 levels, a hierarchical analysis of molecular variance from a matrix of squared distances between all pairs of haplotypes, and calculates variance components and Φ-statistics (haplotypic correlation measures) for each level of population subdivision (*e.g.* "within social groups", "among social groups/within

oceans", and "among oceans"). Φ-statistics are analogous to F-statistics (Wright, 1965) but allow for the haploid transmission of maternal genomes. AMOVA has advantages over traditional F_{ST}, as this program uses the actual sequence data of the haplotypes to obtain interhaplotypic distances. Thus, there is no assumption that all haplotypes are equally distant. Significance was evaluated using permutational analysis of the null distribution (1000 permutations) for each variance component, which gives the probability of a more extreme variance component or Φ-statistic than the observed value. This method of significance testing does not assume that the data are independent and normally-distributed. As recommended by the authors of the program, we used a standard Euclidean matrix of the number of sequence differences between all pairs of haplotypes.

b. *Sequence Similarity Analysis (SSA)*

In addition to the AMOVA, I used a BASIC program written by Hal Whitehead that examined the similarity of mtDNA sequences among pairs of whales in the same social group or in different groups. For whales in different social groups, similarity was examined at the levels of (1) same place, (2) same area but different places, (3) same ocean but different areas, and (4) different oceans. This program calculated the mean number of sequence changes between all pairs of individuals, and estimated standard errors using jackknifing, in which the analysis was repeated omitting each sample in

turn (Sokal and Rohlf, 1981). In this analysis, all samples from mature males (easily identified in the field due to their larger size) were excluded.

Analyses (both AMOVA and SSA) were done on two datasets, the first (N=182) in which all whales were included (there is a possibility of some samples being repeated) and the second (N=158) which was edited, using photoidentifications, field observations and data from microsatellite assays, to exclude possible duplicates. Analysis of both datasets produced similar results, with no changes in the significance of any value. Only the results from the inclusive dataset are presented here.

Results

5.5 Definition of haplotypes

Mitochondrial control region sequences were obtained from sperm whales in the Pacific (N=140), Atlantic (N=23) and Indian (N=19) oceans. Of the 500-600 bp sequenced, 12 variable nucleotide positions were identified. All were transition substitutions. These variable positions defined 12 mtDNA haplotypes (Table 5.1). The most divergent mtDNA haplotypes differed at 9 of the 12 variable sites. The phylogenetic relationship among the haplotypes, obtained using the maximum parsimony algorithm of PHYLIP v. 3.4 (Felsenstein, 1981), is shown in Fig. 5.2. This tree was rooted with the sequence from the pygmy sperm whale control region (M. Dillon, unpublished data).

Table 5.1: Mitochondrial control region haplotypes found in sperm whales. Positions are given relative to the sequence shown in chapter 3.

position	58	62	105	184	200	208	211	261	273	289	320	325
substitution #	1	2	3	4	5	6	7	8	9	10	11	12
Haplotype 1	T	C	C	T	T	A	C	A	A	A	G	C
Haplotype 2	T	T	C	T	T	A	C	A	A	G	G	C
Haplotype 3	T	T	C	T	T	A	C	A	A	A	G	C
Haplotype 4	T	T	C	T	T	A	C	A	A	A	A	C
Haplotype 5	T	T	C	C	T	A	C	G	A	A	A	T
Haplotype 6	T	C	C	T	T	A	C	A	A	G	G	C
Haplotype 7	C	C	C	T	T	A	C	A	A	A	G	C
Haplotype 8	T	C	C	T	T	G	C	A	A	A	G	C
Haplotype 9	T	C	C	T	T	A	C	A	A	A	G	T
Haplotype 10	T	C	C	T	T	A	C	A	G	A	G	C
Haplotype 11	T	C	C	T	C	A	T	A	A	A	G	C
Haplotype 12	T	T	T	C	T	A	C	G	G	A	A	T

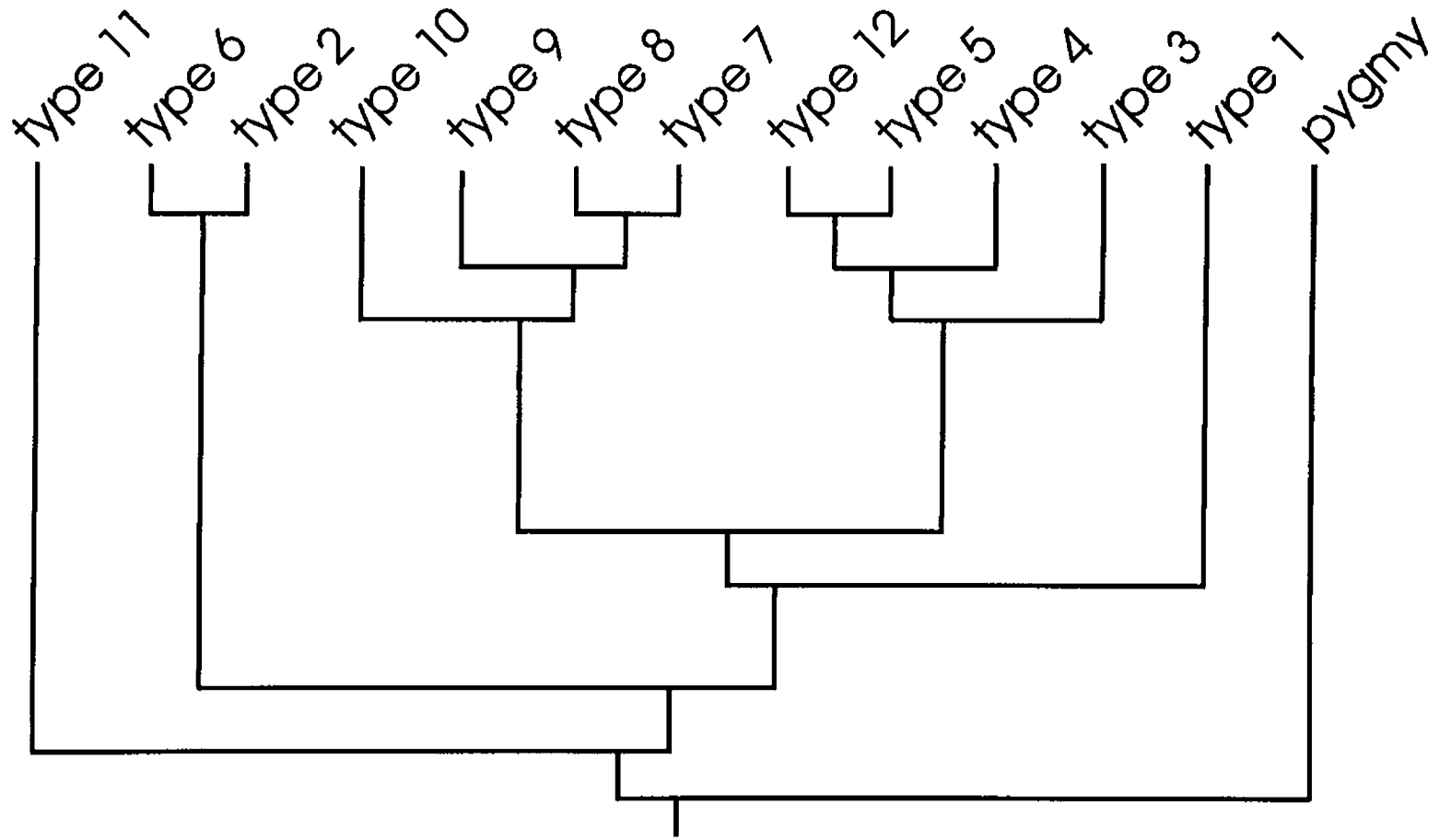


Figure 5.2: Phylogenetic relationships among the 12 sperm whale haplotypes rooted with the sequence from the pygmy sperm whale. Tree was obtained using the maximum parsimony algorithm of PHYLIP (Felsenstein, 1991).

5.6 Structure of groups

Twenty-five groups of whales showing behavioural coordination were examined in this study. The number of individuals sampled within a group ranged from 2 to 19, with a mean of 7. Each group was dominated by one mtDNA haplotype. On average, the dominant haplotype in a group had a frequency of 79% (range of 29 to 100%). Sequence similarity analysis revealed that individuals within groups had fewer changes in the control region (mean of 1.0 difference) than individuals in the same place in the ocean but in different social groups (mean of 1.5; Fig. 5.3). Thus it seems that groups are composed of related individuals, and, based on the predominance of one maternally-inherited mtDNA haplotype in each group, have a matrilineal structure.

Because of the relatedness of group members, I considered the group from which an individual came in the analysis of molecular variance at different hierarchical levels.

5.7 mtDNA Variation over broad geographic scales

There was no obvious geographical structure overlying these genetic differences between groups, either at the scale of areas within the S. Pacific, from which we have most samples and observational data, or at the very broad scale of 3 major oceans. I first examined the S. Pacific alone for evidence of genetic differences in 3 widely-separated geographical areas (Fig. 5.4). Based on matching of fluke identification photos throughout the S. Pacific to those from

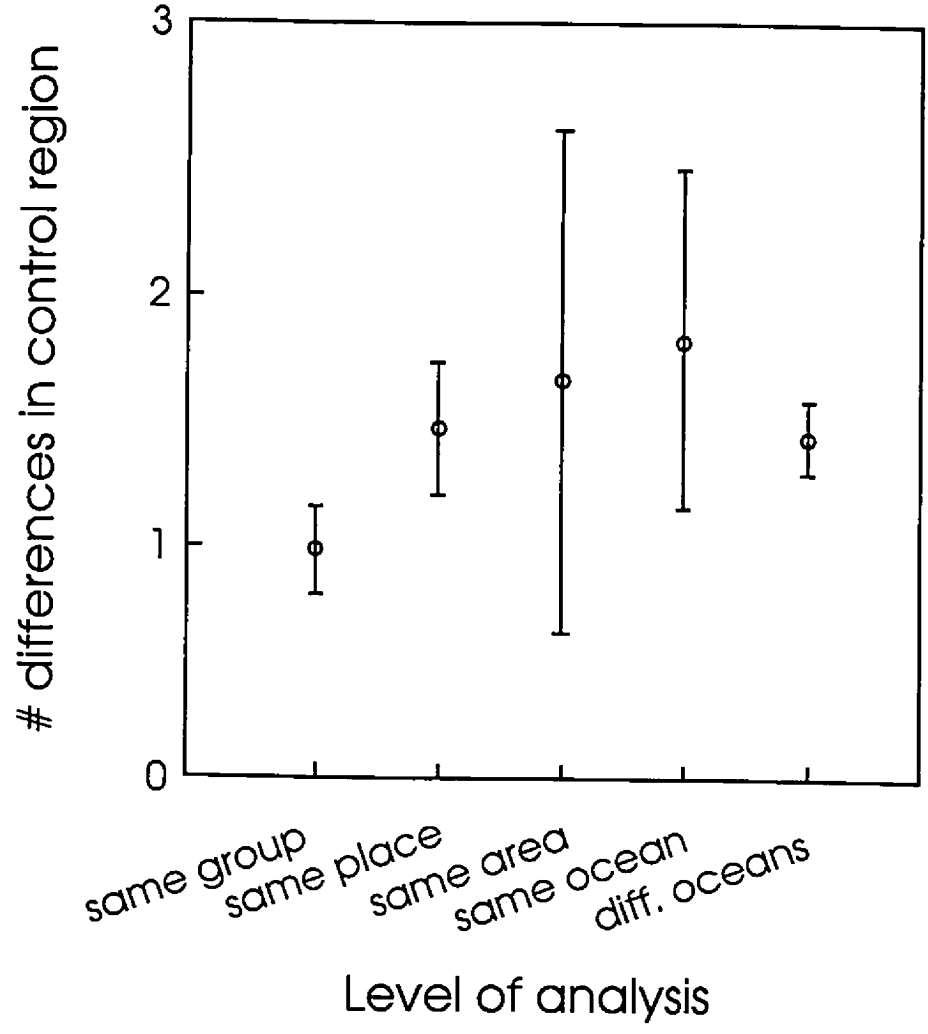


Figure 5.3: Mean number of sequence differences in the mitochondrial control region between individuals in the same social group, and those in different social groups but in the same place, same area, same ocean and different oceans. Vertical bars represent ± 1 jackknifed standard error.

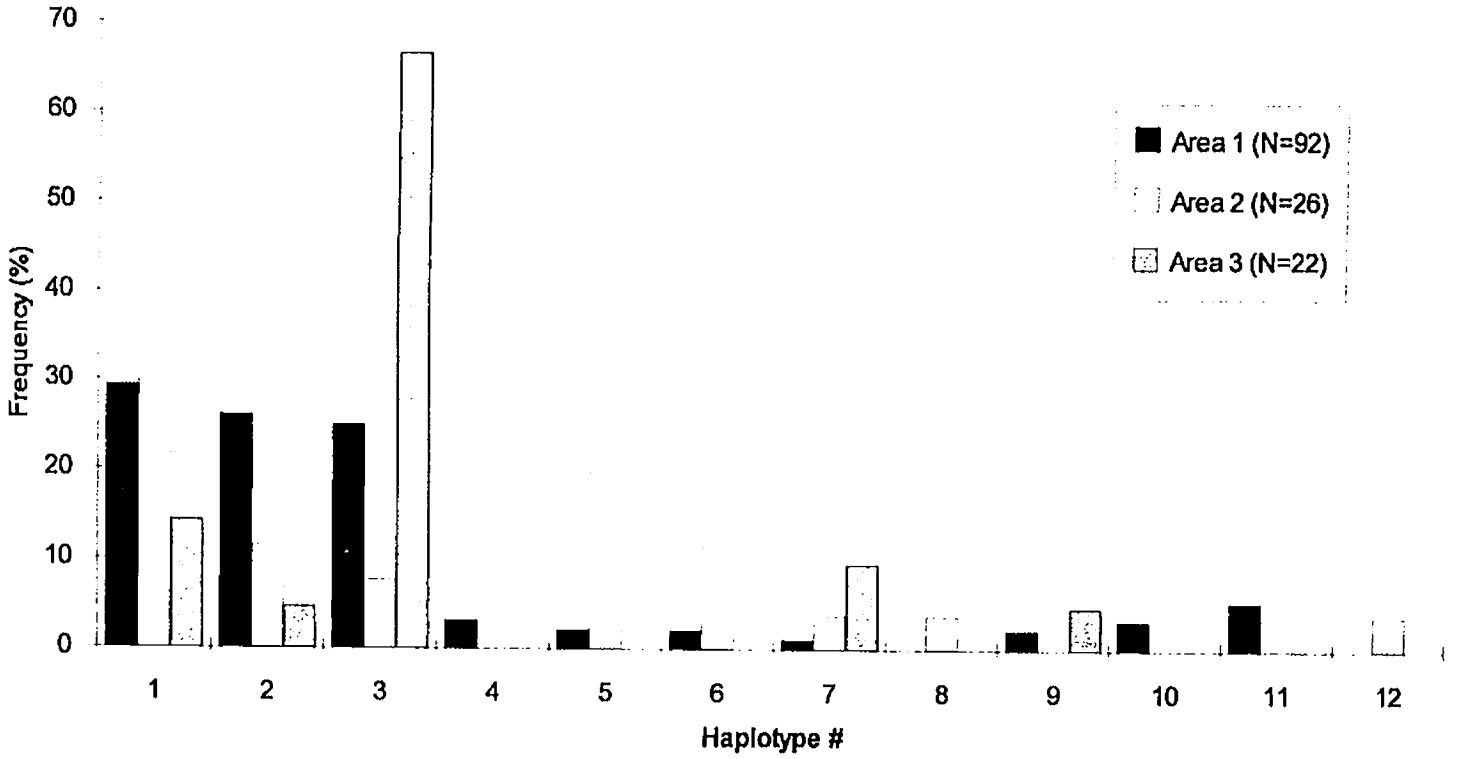


Figure 5.4: Frequencies of mitochondrial haplotypes in 3 areas in the S. Pacific.

the Galápagos and mainland Ecuador, Dufault and Whitehead (1995) proposed that these areas (Galápagos/Ecuador/N. Peru; W. Pacific; and South Pacific south of 10°S) represented geographically distinct stocks. The results of the AMOVA for these areas revealed that none of the total variance in the S. Pacific could be accounted for by among-area differences; 33% was due to differences among social groups within areas, while the remaining 67% was accounted for by variance within social groups (Table 5.2). The mtDNA sequence results, therefore, do not suggest that the areas are genetically distinct.

Similarly, the distribution of haplotypes in three oceans (Fig. 5.5) did not show obvious geographical differences. There were haplotypes sampled in the Pacific that were not observed in the other oceans, but the haplotypes common in the Atlantic and Indian Oceans were also common in the Pacific. Again, the analysis of molecular variance at all levels (*i.e* groups within places; groups within areas; groups within oceans) revealed that very little, if any, of the overall variance was due to structuring at higher geographic levels (Table 5.3).

Discussion

5.8 Social organization of female sperm whales

The predominance of one mtDNA haplotype in a group and the smaller number of sequence differences between individuals in the same, as opposed to different, social groups suggests that grouped females are often relatives, and are consistent

Table 5.2: Hierarchical analysis of molecular variance of sperm whale mtDNA haplotypes from 3 widely-separated areas in the S. Pacific ocean.

Social groups within 3 S. Pacific areas

Variance:	df	% total variance	Φ	p[1]
Among areas	2	-0.01[2]	CT= 0.000	0.545
Among social groups / areas	23	33.30	SC= 0.333	0.001
Within social groups	114	66.70	ST= 0.333	0.001

1. Significance was determined by testing variance components against a null distribution, using 1000 random permutations of the data matrix. The p value is the probability of a more extreme variance component or Φ-statistic than the observed value. For example, the null distribution to test Φ_{CT} and the variance among areas assumes the groups are real but the areas are artificial, permuting groups across areas. The permutation scheme for Φ_{SC} and variance among groups within areas assumes the areas are real, but the groups in them are not, and permutes individuals within areas without considering which group the individual is from. Φ_{ST} and the variance of individuals within groups is tested by allocation of each individual to a random group, holding sample sizes to the real values.

2. Small, negative molecular variance components may occur at the higher hierarchical levels (see Excoffier et al., 1992).

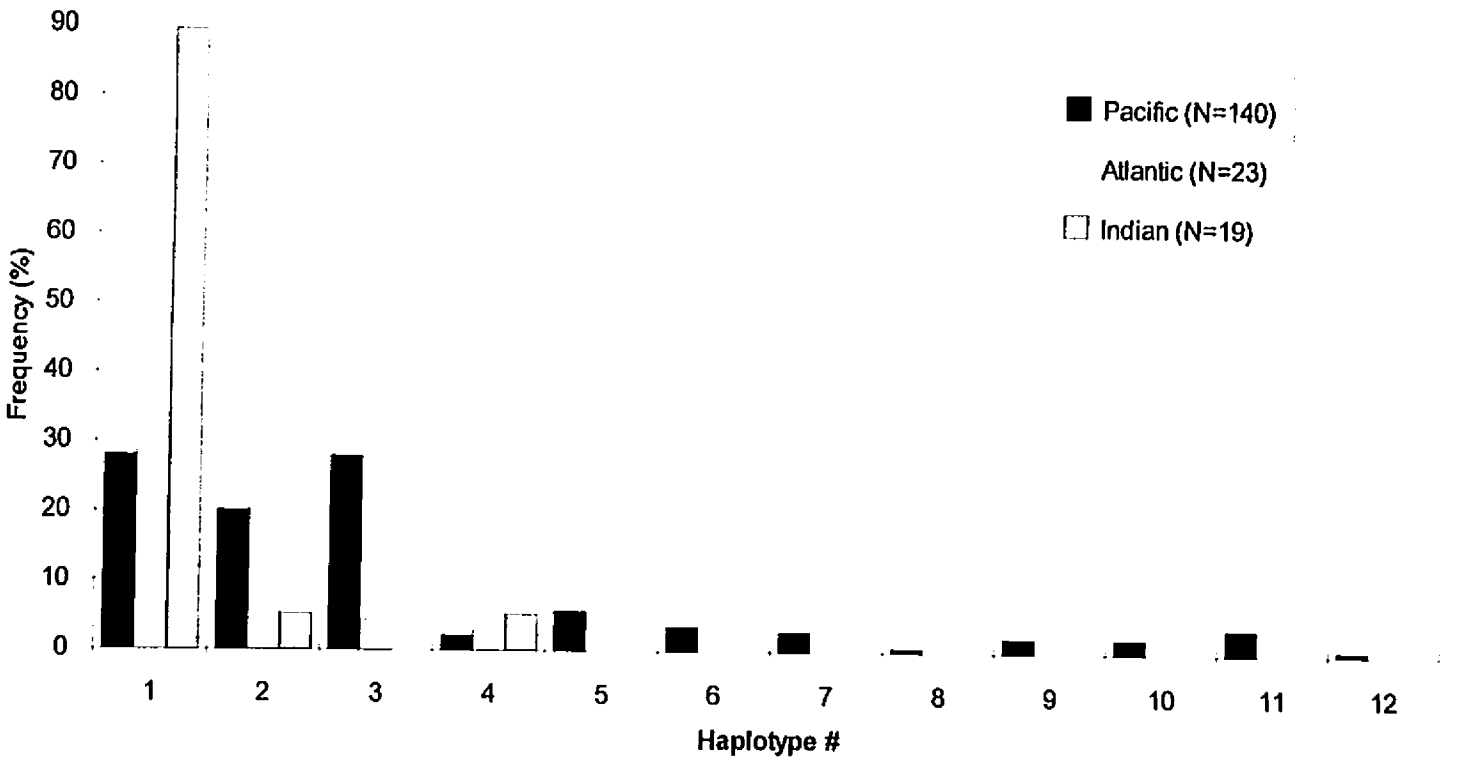


Figure 5.5: Frequencies of mitochondrial haplotypes in the Pacific, Atlantic and Indian oceans

Table 5.3: Hierarchical analysis of molecular variance of sperm whale mtDNA haplotypes from the Pacific, Atlantic and Indian oceans.

Analysis 1: Social groups within places

Variance:	df	% total variance	Φ	p
Among places	14	2.70	CT= 0.027	0.42
Among social groups / places	18	38.36	SC= 0.394	0.001
Within social groups	149	58.94	ST= 0.411	0.001

Analysis 2: Social groups within areas

Variance:	df	% total variance	Φ	p
Among areas	8	4.65	CT= 0.047	0.24
Among social groups / areas	24	36.90	SC= 0.387	0.001
Within social groups	149	58.45	ST= 0.416	0.001

Analysis 3: Social groups within oceans

Variance:	df	% total variance	Φ	p
Among oceans	2	-2.85	CT=-0.029	0.56
Among social groups / oceans	30	42.69	SC= 0.415	0.001
Within social groups	149	60.16	ST= 0.398	0.001

with the groups being composed of matrilines. This study supports the findings presented in chapter 4, which examined 3 groups in detail using both microsatellite and mitochondrial markers, and expands on it by surveying many more groups over a wide geographic range.

5.9 Movement of female sperm whales within and among oceans

Given that studies of the movement of sperm whales using artificial tags (Ivashin, 1977; Ivashin 1981) and natural fluke markings (Dufault and Whitehead, 1995) suggest very limited movements of females, it was surprising to find apparent genetic uniformity of mtDNA haplotypes within and among oceans. This situation in sperm whales is also in contrast to that of some other cetaceans. For example, Baker *et al.* (1994) examined mtDNA haplotypes in humpback whales (*Megaptera novaeangliae*). Haplotypes were derived from restriction fragment length polymorphisms of the entire mtDNA molecule digested with 5 restriction enzymes. The authors reported that not one of the 22 haplotypes observed was common to the three oceanic populations examined (North Pacific, North Atlantic and Southern oceans). For the sperm whales, 3 of the most common haplotypes were found in all 3 oceans, and another occurred in 2 oceans. The remaining less common haplotypes were found only in the Pacific ocean, which probably reflects the larger sample analyzed from the Pacific compared to the Atlantic and Indian oceans. My results are, however, similar to those found in Dall's porpoise

(*Phocoenoides dalli*), where 3 of 4 abundant haplotypes were distributed widely across the North Pacific sampling area (McMillan and Bermingham, 1996).

Completely restricted movement of female sperm whales among areas within oceans and among oceans might be expected to result in differentiation of the maternally-inherited mtDNA molecule over time. It is worth noting that such time scales are difficult to assess, as divergence rates for mtDNA control region sequences vary substantially across taxa, with the rate in some cetaceans being relatively slow (Hoelzel *et al.*, 1991). An observed lack of differentiation does not, however, necessarily mean extensive mixing of females from different areas or oceans, as one migrant female per generation is enough to prevent differentiation (Slatkin, 1987). Movement of female sperm whales may be limited, as indicated by marking studies, but sufficient to produce the uniformity of mtDNA haplotypes observed in this study. This homogenizing effect may be especially pronounced, given an apparent reduction of mtDNA variability in sperm whales (see below).

Alternatively, it is possible that, despite observations of genetic uniformity, there could be a complete lack of migration of female sperm whales among oceans at present if there had been movement in the past. The substantial whaling that sperm whales have been subjected to in the past could have precipitated migration of female sperm whales. For example, Kahn *et al.* (1993) have suggested that sperm whales

move from areas of low exploitation to regions of high, recent exploitation. If the movement of whales was recent enough that populations have not diverged, both uniformity of mtDNA haplotypes and a lack of movement of marked females could be observed. In terms of management, it is important to realize that indications of movements (*i.e.* uniformity of mtDNA haplotypes) do not necessarily mean that there is migration. To gain more insight into genetic differentiation and gene flow in sperm whales, more research is required, using both direct methods (observations of marked whales) and indirect approaches (inferences derived from genetic studies).

5.10 Implications for conservation and management

An important implication of this study is that females that travel together and stay together over time are closely related. Since this relatedness forms the basis for the sociality that characterizes groups of sperm whales, consideration of these social groups will be important in decisions involving management of the species (*e.g.* Best, 1979; IWC, 1986). An additional consideration for management decisions relates to the amount of variability seen in sperm whales in my sample. I was surprised to see the lack of obvious differentiation of mtDNA haplotypes among the Pacific, Atlantic and Indian Oceans. There were only 12 variable positions observed in the most variable part of the sperm whale mtDNA control region in a world-wide sample of 182 whales in this study. By contrast, Baker *et al.* (1993) found

33 variable positions in the same region from 90 humpback whales. The apparent reduced amount of variability in sperm whales could indicate that past hunting has reduced the genetic variation in this species. Some evidence supports this hypothesis. Lyrholm *et al.* (1995) have preliminary data based on control region sequences from 40 sperm whales sampled over a wide global range. They report that the nucleotide diversity in the sperm whale is similar to populations known to have experienced bottlenecks, and several times lower than reported for abundant and outbred populations. Further investigations into genetic variability in sperm whales clearly are necessary, but the results presented in this chapter are indicative of a lack of mtDNA variation among sperm whales sampled world-wide.

Summary

Analysis of mtDNA control region sequences in social groups of sperm whales showed that groups were composed of related individuals, and based on the predominance of one haplotype in each group, suggested that groups have a matrilineal structure. At geographic levels above that of the group, however, very little structure was seen. The DNA sequences from the sperm whale control region examined here seemed surprisingly non-variable.

CHAPTER 6: General Discussion

Working from DNA derived from sloughed skin, I used sequences from the mitochondrial control region to examine some of the attributes that make sperm whales so interesting.

Sloughed skin was found to be a good source of DNA for this work. Like most aspects of molecular biology, this has its advantages and disadvantages, however. It is advantageous in that many samples can be collected quickly (for example, we collected over 20 samples from "group C" in about 30 minutes). Also, this method of sampling fits well into our research, in that both fluke identifications and skin samples can be taken simultaneously, without having to prioritize collection of different data. Obviously, sampling completely non-invasively is better for the whales, and probably also for the collection of behavioural data. Finally, this sampling approach is entirely straightforward, in that no permits, nor special skills (*e.g.* crossbow or rifle expertise) are required.

The major drawback of using sloughed skin is that the DNA in these samples is degraded. Skin from the head and back of the whale has probably had considerable exposure to ultraviolet rays, potentially damaging the DNA. Poor sample quality was seen in a relatively high failure rate of PCR experiments for both mitochondrial DNA and microsatellites (K. Richard, personal communication). I estimate that 30-40% of PCR reactions failed and in virtually all cases, template

quality seemed to be the problem. Such a failure rate seems higher than comparable work using better quality tissue (non-destructive samples such as biopsies, as well as heart and muscle tissue).

Another disadvantage of working from sloughed skin is that it is not possible to get samples from specific whales on demand. This is a drawback when it would be desirable to link a genetic sample to a particular whale, for example, if 2 adults were seen accompanying a calf. The majority of sloughed skin samples we obtained were not linked to identified individuals, having been collected in the wake of groups of whales. Many samples could be from the same whale, and to test this genetically requires considerable time and effort (*e.g.* microsatellite assays at sufficient loci to distinguish individuals). Biopsy samples from sperm whales also have the disadvantage of not always being linked to fluke identifications; the difference, however, is that other information (*e.g.* scars, dorsal fin shape etc.) could be used to ensure that the same whale was not repeatedly sampled. Therefore, even though the whale is not individually identified by its fluke markings, it is known to be a distinct individual, and not one that could have been sampled before.

Despite these limitations, the quality of skin samples did not prevent me from addressing any of the questions I set out to investigate. The major effect of poor-quality sloughed skin samples was that the laboratory work was at times slow,

requiring repeated extraction and amplifications, and therefore, sample sizes may be somewhat smaller than those that could have been obtained with high quality tissue.

Analysis of the molecular evolution of the control region was not intended to resolve the debate about position of sperm whales in cetacean evolution (one of the most contentious issues in cetacean biology at the moment), but did provide additional evidence for the sperm whale's unique position among the cetaceans. The finding that certain blocks of sequences that were previously described as being highly conserved in cetaceans were not well conserved in sperm whales calls for re-evaluation of the function of these sequences in the mitochondrial genome.

Investigation into the group structure of sperm whales revealed the genetic relatedness of members of social groups, which has implications for the evolution and maintenance of communal care of calves and other social behaviours observed in groups of female sperm whales. This study was especially interesting in the combination of observational data with different molecular markers. Comparing results from both nuclear and mitochondrial markers provided insight not available with one dataset alone, for example the indications of relatedness among group members with different mitochondrial haplotypes. While this analysis answered some questions about the social organization of sperm whales, it also showed that there remains much to be learned. Our

knowledge of female groups is far from complete, but as this work showed, information on males is even worse (in part because of their scarcity on breeding grounds). Many aspects about the lives of males, including how often they breed, to what extent they fight for access to females, whether and how dominance hierarchies are established and maintained, and how selective whaling for the largest males has affected breeding in this species, remain to be elucidated. Insight into some aspects of the breeding system could be addressed using variable male-specific genetic markers. Such markers would nicely complement the system described in this thesis (maternally inherited and nuclear alleles).

Finally, the survey of mtDNA variation in populations of sperm whales from three major oceans provided further support for the genetic relatedness of members of social groups, but unexpectedly, did not reveal much structure at higher levels (places, areas, oceans). If the geographic ranges of female sperm whales are as limited as suggested from marking studies, there should have been genetic differentiation of mtDNA within and among oceans. The apparent reduced variability in the mitochondrial DNA of sperm whales compared to other cetaceans was especially surprising. Because of this lack of differentiation, I can not say as much about the genetic stock structure as I had hoped. Nevertheless, these results are tremendously important in terms of conservation and management of this species. It may be that sperm whales have gone though

a genetic bottleneck, leading to the lack of variation observed. It is clear that no whaling of this species should occur without a better understanding of how past hunting has affected their genetic variability. Information from more highly variable loci (*e.g.* microsatellites) analyzed over a wide geographic range may be particularly interesting in this regard.

In conclusion, while much remains to be learned about the genetics of sperm whales, this thesis adds to the current state of knowledge. On a molecular level, I demonstrated that sperm whales are notably different genetically, and I show the need to exercise caution when speculating on the importance of sequences conserved among some cetaceans, but not others. On a group level, this work showed the strong patterns of maternal relatedness among members of social groups. A comparison of mitochondrial data to nuclear data revealed interesting and unexpected associations in groups such as paternal relatedness. Finally, on a world-wide scale, I found no structure overlying the strong relatedness of group members, and showed a surprising lack of variation in the mitochondrial control region of sperm whales sampled in three major ocean basins.

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