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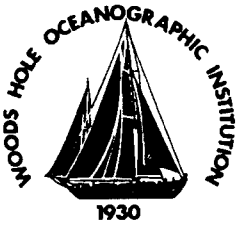

Joint Program in Oceanography/ Applied Ocean Science and Engineering

DOCTORAL DISSERTATION

Mitochondrial DNA Sequence Variation in North Atlantic Long-finned Pilot Whales, *Globicephala melas*

by

Liese Anne Siemann

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Woods Hole Oceanographic Institution
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Funding was provided by a New Initiative grant through the National Oceanic and Atmospheric Administration National Sea Grant Program (Grant no. NA86-AA-D-SG090).

Approved for publication; distribution unlimited.

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Mitochondrial DNA Sequence Variation in North Atlantic Long-finned Pilot Whales, *Globicephala melas*

by

Liese Anne Siemann

B.A., Cornell University
(1988)

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

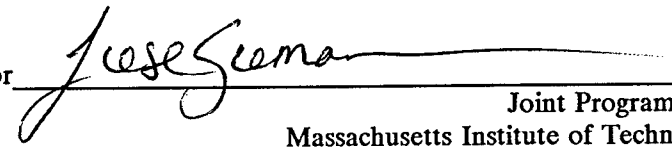
at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION

May 1994

Signature of Author 

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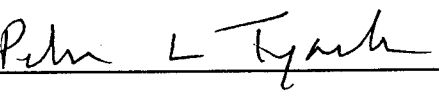
Certified by 

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Mitochondrial DNA Sequence Variation in North Atlantic Long-finned Pilot Whales, *Globicephala melas*

by

Liese Anne Siemann

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution

ABSTRACT

I sequenced mitochondrial DNA (mtDNA) from 59 long-finned pilot whales (*Globicephala melas*) that stranded on the coasts of Cape Cod, Newfoundland, Nova Scotia, Scotland, and England or were caught by commercial fisheries operating in the western North Atlantic, to determine if there is more than one genetic stock in the North Atlantic. Samples from 11 Atlantic and 2 Pacific short-finned pilot whales *(G. macrorhynchus)* and 11 bottlenose dolphins *(Tursiops truncatus)* were also analyzed. Sequences were obtained from 400-bp of the D-loop, a non-coding region in volved in replication, and from 303-bp of the protein gene coding for cytochrome *b*.

The D-loop sequences determined from 55 of the long-finned pilot whales were completely identical. Only the 2 sequences from Canadian whales showed some variability, differing from the other sequence by 0.25 - 0.50% (pairwise sequence divergence). All of the Atlantic short-finned pilot whales had identical D-loop sequences, and this sequence differed from the long-finned pilot whale sequences by 3.25 - 3.75%. The two Pacific short-finned pilot whale sequences differed from each other by 0.25%, from the Atlantic short-finned pilot whale sequence by 0.25 - 0.50%, and from the long-finned pilot whale sequences by 2.75 - 3.50%. D-loop nucleotide diversity in long-finned pilot whales was 0.03% and in short-finned pilot whales was 0.05%. The cytochrome *b* gene sequences determined for 16 long-finned pilot whales from all sampled locations, 4 Atlantic short-finned pilot whales, and two Pacific short finned pilot whales were all identical within each group, and differed from each other by 0.33 - 0.99%. Finally, D-loop sequences were also determined from 11 bottlenosed dolphins. All of the individuals had distinct D-loop sequences that differed by 0.25 - 4.25%, and the nucleotide diversity was 1.25%. Two dolphins were caught together, and the sequence divergence within this pair was 3.50%.

These results suggest that long-finned pilot whales from the eastern and western North Atlantic are not genetically isolated from each other and that mtDNA variability in pilot whales may be unusually low. This might be a result of a slow rate of sequence evolution or metapopulation dynamics resulting from the social system of pilot whales.

To examine the effect of social structure, I used an individual-based model designed to study the effect of sub-population extinction on mitochondrial genetic diversity in a pilot whale population which is subdivided as a result of this species' social system. MtDNA diversity was monitored in a population of pilot whales when extinction rates, mutation rates, and pod dynamics were altered. The results of the simulations indicate that if a pilot whale population experienced a moderate level of pod extinctions, it could undergo large fluctuations of mtDNA heterozygosity over time and frequently have the low heterozygosity observed in the North Atlantic pilot whale population.

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ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor, Peter Tyack, for his advice and his excitement about my research during the last six years, and I would like to thank my committee members for all of their suggestions and encouragement along the way - Solange Brault, Hal Caswell, Mary Lou Pardue, Chris Simon, and Tim Smith. I need to express my appreciation to Judy McDowell, who acted as chair for my oral exam and my defense. Special thanks must also go to Peter and Judy for their support when my life in and out of graduate school became complicated.

I must acknowledge all of the people and institutions that supplied tissue samples, for without their support and cooperation, this project never would have happened. The following institutions supplied most of the samples used in my research - the New England Aquarium, the National Marine Fisheries Service, and the Smithsonian Institution. My sincere thanks go out to the individuals at these instituions and others that supplied additional samples - Greg Early and his staff at the New England Aquarium, Tim Smith and John Nicolas at the Northeast Fisheries Sciences Center, Bill Perrin and Rick LeDuc at the Southwest Fisheries Sciences Center, Jim Mead and Charlie Potter at the Smithsonian Institution, Bill Amos at the University of Cambridge, Harry Ross and Bob Reid at the Inverness Veterinary Investigation Centre, Kenny Richard at Dalhousie University, and Jon Lien and Kristina Curran at Memorial University.

I would also like to thank the people in the labs I worked in during graduate school. Special thanks must go to Kurt Fristrup, my collaborator on the modelling project - without his tireless enthusiasm for my ideas and his willingness to take the time to write the program, the model certainly would not have been as interesting as it turned out to be. I must also express my appreciation to Doug Prasher, in whose lab my molecular research began, and to Bonnie Woodward, who taught me the basics of DNA sequencing and who was always a real pleasure to be around during hours at the bench.

On a more personal note, I would like to thank my family for their love and support -my parents, Hannah and Robert, and my brother and sisters, Evan, Gaby, and Fran. My father wouldn't let me give up four years ago, and my mother never stopped thinking I could do anything. I also need to thank my husband Dale for his all of his love and support - during the time we've been together, he has been my rational half, helping me look beyond all of my temporary setbacks. Finally, I want to thank my son Jasper, whose beautiful smile was a light in my life during this last difficult year.

I had the pleasure of making many new friends during graduate school. Together, they made graduate school enjoyable, and I will truly miss having them nearby. A few will undoubtably be friends for life - Cheri, my office-mate and confidant, Andrea, my housemate for three years, and David, with whom I shared the trials and tribulations of DNA sequencing.

There are a few other individuals who need to be mentioned. I would like to thank the people in the Education Office - Jake Peirson, John Farrington, and Abbie Jackson in particular - for their advice and help with all of the details involved with being in graduate school. I would like to thank Tom Kocher for meeting with me and giving me the hints I needed to sequence PCR products successfully. R. Natalie Goodall supplied some rare tissue samples from Peale's dolphins that I did not end up using for my research.

My research was funded by a New Initiative grant through NOAA National Sea Grant (grant no. NA86-AA-D-SG090) and by an Ocean Ventures Fund grant through the Education Office. Tuition and stipend support were supplied by a National Science Foundation Graduate Fellowship and by a research assistantship through the Education Office.

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CHAPTER ONE

Introduction to the North Atlantic Long-finned Pilot Whale and Mitochondrial DNA Analysis

INTRODUCTION

During recent years, the North Atlantic long-finned pilot whale, *Globicephala melas*, has received increased consideration from government and conservation agencies. North Atlantic pilot whales are incidentally taken by commercial fisheries, primarily the foreign and domestic mackerel trawl fisheries, operating in US waters (Douglas 1989, Waring et al. 1990) and are or have been hunted in parts of their range (Andersen 1988, Mitchell 1975a, 1975b, Sergeant 1962). In the western North Atlantic, incidental fishery mortalities from 1977 to 1988 were greater than 297 whales (Waring et al. 1990). In addition, a drive fishery continues to operate in the Faroe Islands in the eastern North Atlantic. This fishery has taken hundreds to thousands of whales per year for centuries, and in recent years, annual catches of greater than 1500 whales have not been uncommon (International Council for the Exploration of the Sea 1993, International Whaling Commission 1990). Additional losses occur during mass strandings (Sergeant 1982). Although the long-finned pilot whale is not considered an endangered species at present, it is a protected species under the US Marine Mammal Protection Act of 1972 (MMPA).

In order to tackle the problem of managing the North Atlantic pilot whale, it will be necessary to understand its genetic structure. The MMPA provides protection for species and population stocks of marine mammals. By providing protection for population stocks, defined as "a group of marine mammals of the same species or smaller taxa in a common spatial arrangement, that interbreed when mature", the US government recognized the importance of preserving the genetic structure of populations (Bean 1983). Ideally, management policies would result in the protection of stocks that have genetic uniqueness and supply genetic variation necessary for the health and survival of the species (Ryder 1986). Variation of neutral genetic markers is one source of information which can be used to define stocks (Dizon et al. 1992, Hoelzel & Dover 1989, Lande 1991), and molecular biological techniques now provide methods which can be used to analyze neutral genetic markers and study the genetic structure of marine mammal populations with small tissue samples (for review, see Hoelzel & Dover 1989). Throughout, unless otherwise stated, the term "population" will be used to refer to a geographically defined group within a species. The term "stock" will be used to refer to a genetically adapted and differentiated population within a species (a genetic stock).

My dissertation research focused on answering two questions:

1) Can mitochondrial DNA (mtDNA) sequence variation be used to help define genetic stocks of pilot whales in the North Atlantic?

2) Is mtDNA sequence variation affected by the social structure of pilot whales?

MtDNA analysis has been recommended for the assessment of marine animal stocks (Ferris & Berg 1987, Ovenden 1990) and has been used to study the genetic structure of cetacean populations (studies reviewed in later sections). It has also been shown that the behavior and movements of females can affect patterns of mitochondrial genetic variation (Bowen et al. 1992).

Some Measures of Genetic Variation

The measures of genetic variation used in the studies described in this chapter include:

1) Genetic distance (d, p, δ, or D) is a measure of the number of nucleotide substitutions per site between 2 sequences, and it can be estimated in a number of ways from the proportion of shared restriction fragments (Nei & Li 1979, Upholt 1977) or the proportion of different nucleotides between the 2 sequences (Brown et al. 1982, Hillis & Moritz 1991, Kimura & Ohta 1972, Nei 1987). The genetic distance between 2 populations can be obtained by averaging the pairwise genetic distances between sequences from the 2 populations and correcting this value by the average pairwise genetic distance between sequences from within the 2 populations.

2) Nucleotide diversity (π) is a measure of the extent of DNA polymorphism at the nucleotide level in a population. It can be estimated from the proportion of different nucleotides between different pairs of sequences in the population and the frequency of the sequences in the population giving a measure of the average number of nucleotide differences per site in the population (Nei 1987).

3) Gene diversity or heterozygosity (h, $0<h<1$) is a measure of the extent of DNA polymorphism at the gene level, or the probability that genes (or sequences) from 2 randomly chosen individuals in a population are different. It can be estimated from the frequency of gene (or sequence) types and the number of individuals sampled (Nei 1987).

Details on the calculations used to obtain these estimates of genetic variation can be found in the appendix.

BACKGROUND INFORMATION ON PILOT WHALES

Natural History

The long-finned pilot whale inhabits subpolar and temperate waters in the northern and southern hemispheres. In the northern hemisphere, its distribution is limited to the North Atlantic, while in the southern hemisphere, it has a circumpolar distribution (Figure 1). The warm temperate and tropical waters lying between the northern and southern long-finned pilot whale populations are inhabited by a similar congeneric species, the short-finned pilot whale, *G. macrorhynchus* (Figure 1). The two species may coexist in parts of their ranges, or they may use the same range during different seasons. For instance, the southern boundary of the range of long-finned pilot whales in the western North Atlantic is Cape Hatteras (latitude 35°N), while short-finned pilot whales have been sighted as far north as Delaware Bay (latitude 39°N) in the western North Atlantic (Sergeant 1982). Long-finned and

short-finned pilot whales differ morphologically in the length of their pectoral fins and in the number of teeth in their lower and upper jaws. The social behavior and reproductive biology of short-finned pilot whales have been intensively studied, and this knowledge about short-finned pilot whales might be used to gain insight into life history characteristics of long-finned pilot whales. However, any cross-species comparisons must be interpreted with caution because it has been suggested that the reproductive biology and social structure of the two species may differ (Kasuya et al. 1988). For instance, female short-finned pilot whales appear to have a post-reproductive phase which can account for up to 25-30% of their lives (Marsh & Kasuya 1984). However, no post-reproductive females were identified among the long-finned pilot whales whales studied by Sergeant (1962).

Pilot whales are sexually dimorphic. Males reach lengths of up to 6 meters, while females rarely reach lengths of more than 4.5 meters. Females become sexually mature around the age of 6 or 7 and may live to be over 50 years old. Males mature much more slowly, reaching sexual maturity after 12 years, and they can live to be 36 - 40 years old (Kasuya et al. 1988, Martin 1987, Sergeant 1962). Pilot whales are sighted in groups which range in size from 10 - 1000 individuals. The composition of the smaller groups, or pods, of short-finned pilot whales appears to remain stable for periods of months to years (Dan McSweeney personal communication, Shane 1985). The large groups may be made up of pods which associate for short intervals for mating or feeding, as is the case in killer whales (*Orcinus orca*) in Puget Sound (Bigg 1982).

Genetic analysis of long-finned pilot whales (described in a later section) has indicated that pods seem to consist of a core of related females with their immature and mature offspring and that both sexes remain with their natal pods (Amos et al. 1991a, 1991b, 1993). In addition, because this genetic analysis indicated that calves are not fathered by males in their pods, reproductively active males must move between pods to mate (Amos et al. 1993). Pods in which there were no adult males and pods in which up to fifty percent of the mature animals were male have been reported (Sergeant 1962, Shane 1985). Although groups of pilot whales containing only immature and mature males might be expected to account for the deficit of males, no such groups have been reported for long-finned pilot whales. Because the sex ratio of long-finned pilot whales at birth appears to be roughly even (International Whaling Commission 1990, Sergeant 1962), male long-finned pilot whales appear to have a higher mortality rate than females. The high degree of sexual dimorphism, the sex biased mortality rates, and the skewed sex ratios within herds all suggest that pilot whales are polygynous (Fedigan 1992).

Population Statistics for North Atlantic Long-finned Pilot Whales

Although the size of the entire North Atlantic population is not known, there are estimates of pilot whale abundances in different regions of the North Atlantic. Data from sighting surveys in the eastern North Atlantic in 1989 have been used to estimate abundances of approximately 778,000 whales (CV=0.295) in an area which ranged from the eastern coast of Greenland and Iceland to the northern coast of Spain (Buckland et al. in press). In the western North Atlantic, Mercer (1975)

estimated that there were 60,000 pilot whales in the population found in Newfoundland waters in 1947, before the Newfoundland drive fishery took 54,348 whales from 1947 to 1972, and data from aerial surveys conducted off Newfoundland and Labrador in 1980 gave abundance estimates of 6700 - 19,600 whales (International Whaling Commission 1990). Aerial and vessel surveys conducted by the Northeast Fisheries Science Center and the Cetacean and Turtle Assessment Program from 1978 to 1982 yielded abundance estimates of approximately 10,000 - 12,000 whales on the continental shelf off the northeast coast of the United States (International Whaling Commission 1990, Waring et al. 1990). Pilot whales have been sighted in the central North Atlantic, although no abundance estimates are available (International Whaling Commission 1990).

At present, the stock structure of North Atlantic long-finned pilot whales has not been resolved. However, there are distributional data indicating that the western and eastern North Atlantic populations may be distinct. Mitchell (1975b) noted that the crash of the Newfoundland population of pilot whales due to overfishing was not observed in the population around the Faroe Islands, indicating that the Newfoundland and Faroe Island populations were separate stocks. A comparison of characteristic permanent scars caused by nematodes and cysts of cestodes which remain for the life of their hosts has indicated that pilot whales do not move between Newfoundland waters and the eastern North Atlantic (International Whaling Commission 1990). Finally, research on stable isotope ratios in tissues from whales taken along the U.S. coast and in the Faroe Islands suggest that the short-term diets of whales in these areas differ (Abend et al. 1993)

Phenotypic data also suggests that western and eastern North Atlantic pilot whales may be from different stocks. Martin et al. (1987) compared length-at-age values between whales from the Newfoundland fishery (Sergeant 1962) and whales which stranded on the British coast. Their data suggested that the Newfoundland population attains greater mean maximum body lengths and may be distinct from the population in British waters. More recent morphometric analyses found that male flipper length relative to total body length was greater, skull length was longer, and torso length was shorter for whales from Newfoundland in 1951-1954 than for whales from the Faroe Islands in 1988 and 1992, suggesting that these populations are distinct (Bloch & Lastein 1993). It must be mentioned that both of these studies compared phenotypic data from eastern North Atlantic whales taken or stranded 30 years after the western North Atlantic whales, and the observed differences might be attributed to changes over time in one stock. In addition, these differences might be due to isolation by distance in one stock which had a continuum of values for the described traits from the western to eastern North Atlantic.

There is some information available about the movements of one pilot whale in the North Atlantic. In the summer of 1987, a young male pilot whale which had been rehabilitated by the New England Aquarium was tracked after his release using a satellite monitored tag attached to his dorsal fin (Mate 1989). He was tracked over 7,588 km for 95 days. His daily movements were an average of 80 km, with a maximum daily movement of 243 km.

Exploitation of North Atlantic Long-finned Pilot Whales

Pilot whales have been historically exploited throughout the North Atlantic. Some recorded catch statistics are available for different fisheries in Canada, North America, the Faroe Islands, Greenland, Iceland, and Norway, although none of the available records are comprehensive. All available data on catch statistics are summarized in this section.

In the western North Atlantic, the pilot whales around Newfoundland were overhunted by a drive fishery between 1947 and 1972. A total of 54,348 whales were taken during this period, with an average catch of 2,090 whales and a peak catch in 1956 of 9,794 whales (Mercer 1975, Mitchell 1975a,b). The size of the Newfoundland pilot whale population before 1947 was estimated by Mercer (1975) using three modified Leslie-DeLury population models based on the interactions between pilot whales and squid, their primary prey species in that area, and the models gave abundance estimates of 66,273 - 75,361 whales, 49,142 - 54,806 whales, and 62,516 - 79,592 whales, with an overall mean estimate of 62,432 whales. Although the drive fisheries operating in the US were not as intensive as the Newfoundland fishery, annual catches in the 1800s and early 1900s ranged from 8 to 3,493 whales with an average of 420 whales, and single drives of 1500 and 500 whales were reported in 1884 (Kittredge 1930, McFee 1990, Mitchell 1975a, Spears 1908, Starbuck 1964).

The most extensive drive fishery in the eastern North Atlantic operates in the Faroe Islands. The Faroese have hunted pilot whales for centuries, and catch records have been kept since the late 1500s. Between 1584 and 1883, 117,546 whales were taken in the Faroe Islands (Mitchell 1975a). From 1813 to 1859, the average annual

catch was 1,116 whales, and from 1860 to 1877, the average annual catch was 889 whales (Mitchell 1975a). More recently, from 1940 to 1962, the average annual catch was 1,721 whales, and from 1981 to 1992, the average annual catch was 1,947 whales and catches as high as 2,973 were recorded (International Council for the Exploration of the Sea 1993, Mitchell 1975a). Drive fisheries in Norway, Iceland, and Greenland have also taken pilot whales. In Norway, annual catches between 1938 and 1974 ranged from one to 339 whales with an average of 56 whales (Oien 1991), and in Iceland, recorded annual catches from 1606 to 1990 ranged from a low of 3 whales in 1966 to a high of 2000-3000 whales in 1927 (Sigurjónsson 1993). Finally, annual catches in Greenland from 1929 to 1939 and from 1952 to 1990 ranged from 2 to 415 whales with an average of 81 whales (Heide-Jorgensen & Bunch 1991).

Genetic Studies on Pilot Whales

Nuclear genetic markers, including isozymes and satellite DNA, have been used to study gene flow in the Faroese pilot whale population.

Isozyme electrophoresis

Different forms of polymorphic enzymes (isozymes), including different alleles at an enzyme locus, can be distinguished by their net charge and size. When such polymorphic proteins are run on a gel under the influence of an electric field, they can often be visualized as distinct bands using histochemical staining. Organisms can then be clustered by comparing the frequencies of different isozymes or alleles. One study which examined enzyme polymorphisms in long-finned pilot whales around the

Faroe Islands indicated that there is detectable nuclear genetic variation between some pods of long-finned pilot whales. Andersen (1988) surveyed 27 enzymes in 9 pilot whale pods caught in the Faroese drive fishery and found 3 enzymes which were polymorphic. Esterase phenotypes were scored for 412 whales, mannose phosphate isomerase phenotypes were scored for 628 whales, and superoxide dismutase phenotypes were scored for 621 whales. A multilocus G-test on mean heterozygosity values demonstrated a slight but statistically significant difference in allele frequencies between the pods, although there was no correlation between allele frequencies and geographical location around the Faroe Islands. This result was attributed to pilot whales selectively breeding with members of other pods, resulting in reproductive isolation between some pods.

An electrophoretic study was also done on two morphologically distinct forms of short-finned pilot whales off the coast of Japan. Wada (1988) surveyed 36 enzymes and found 2 polymorphic loci among 204 northern form whales and five polymorphic loci among 167 southern form whales. The estimated value for Nei's genetic distance, D, (Nei 1972) between the two forms was 0.0004. This value represents the pairwise average number of electrophoretically detectable variants per locus and can range from 0 to infinity. Upon comparison to other species, he concluded that the low D value of 0.0004 was appropriate for two local populations. Surprisingly, this D value between two morphotypes of short-finned pilot whales was in the low end of the range of inter-pod D values for long-finned pilot whales he calculated from the gene frequency data of Andersen (1988).

DNA fingerprinting and microsatellite analysis

The nuclear genome contains regions of hypervariable tandem-repetitive DNA sequences, known as satellite DNA. Multilocus DNA fingerprinting is one technique which focuses on the analysis of minisatellite DNA. Minisatellite DNA consists of arrays of tandemly repeated short sequences of less than 65-bp, and it includes a core sequence that can be probed at many different loci (Burke 1989, Jeffries et al. 1985). Patterns of DNA variation detected using this technique can be used to distinguish between individuals. More recently, a technique has been developed to examine microsatellites using the polymerase chain reaction. This type of satellite DNA consists of repeated two or three nucleotide motifs created by slippage during DNA replication or repair, and these regions are hypervariable in length in humans, *Drosophila*, and some cetacean species (Schlötterer et al. 1991, Tautz 1989).

The breeding behavior and social structure of long-finned pilot whales has been examined using DNA fingerprinting and microsatellite analysis. Amos et al. (1991a, 1991b) sampled 193 whales from two complete pilot whale pods, including 34 mother-fetus pairs, and used DNA fingerprinting to investigate the paternity of the 34 fetuses. Using paternity exclusion analysis, they determined that 30 of the fetuses were not fathered by any of the mature males in their pods. In a later study, Amos et al. (1993) typed the same whales using six microsatellites. Using this technique, they found that 33 of the fetuses were not fathered by accompanying males. They estimated the number of mother-offspring pairs within the pods and found that 95% of young whales and 35% of adult whales were accompanied by their mothers. In addition, genotype frequencies were assessed using computer simulations to examine

overall relatedness in pods. Genotype frequency indices observed in the pods were similar to those in simulations that generated pods from single females or related groups of females. Therefore, they concluded that mature males remain with their pods, and that pods are extended families of related whales. Because fetuses were not fathered by males within their pods, they concluded that males mate when they travel to other pods or when pods temporarily join.

MITOCHONDRIAL DNA ANALYSIS

Analysis of mitochondrial DNA (mtDNA) has been the focus of the majority of DNA studies to date on the genetic structure of animal populations and species (for reviews, see Avise et al. 1987, Harrison 1989, Simon 1991, Wilson et al. 1985). Mitochondria are the intracellular organelles responsible for cellular respiration, and they contain circular, double-stranded DNA which codes for the 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) necessary for mitochondrial protein synthesis and for thirteen of the proteins involved in mitochondrial protein synthesis and the electron transport chain (Figure 2). Mitochondrial DNA has a number of characteristics which make it especially suitable for studies on population genetics. Each cell contains roughly 10,000 copies of mtDNA, and this makes isolation of mitochondrial DNA relatively simple. Furthermore, mtDNA genes code for proteins and RNAs necessary for cellular respiration and because many regions within these genes appear to be required for normal function, some sequences within mtDNA are highly conserved between classes of organisms. When these sequences are known in

one species, they can often be used to locate or sequence specific segments of DNA in a wide range of other species (Kocher et al. 1989). Because the mitochondrion appears to be extremely inefficient at repairing replication errors, the overall substitution rate in mammalian mtDNA is roughly 5 - 10 times the substitution rate within mammalian single-copy nuclear DNA (Brown et al. 1982). The rapid evolution of many regions in the mitochondrial genome makes it useful for examining interspecific and intraspecific genetic variation. Finally, because mtDNA appears to be maternally inherited through the egg cytoplasm, it is possible to study maternal lineages using mtDNA. Early studies on mtDNA noted that patterns of variation would reflect the behavior of females (Lansman et al. 1981). Consequently, when the dispersal patterns of males and females are not identical, patterns of nuclear and mitochondrial genetic variation may differ. In species where males disperse farther than females, patterns of mtDNA variation might be found even if surveys using nuclear DNA markers show little or no genetic differentiation.

Most studies of mitochondrial genetic variation have been conducted using restriction enzymes. Restriction enzymes recognize specific short sequences of DNA and cleave the DNA at these sites, and they can be used to detect changes in the sequence of the mitochondrial genome when these changes result in loss or gain of a restriction site. When a series of different restriction enzymes are used, it is often possible to distinguish populations or species on the basis of distinct patterns (haplotypes) of restriction fragments.

Genetic variation can also be examined by sequencing specific regions of mtDNA. Sequencing does not rely on chance discoveries of appropriate restriction

enzymes, and because sequencing has finer resolving power than restriction enzyme analysis, it is useful for studying populations which cannot be differentiated using restriction enzymes. DNA sequences can be obtained from regions which have been cloned into appropriate vectors or from enzymatically amplified products of the polymerase chain reaction (PCR). During PCR, specific regions of DNA are amplified when two short oligonucleotide sequences, commonly known as primers, anneal to specific regions of DNA on opposite strands and are extended in an enzyme-mediated reaction toward each other. Analysis of PCR products is a very valuable technique because it can be done when only extremely small tissue samples are available.

The D-loop

The region within mtDNA commonly thought to be most variable is the D-loop, a non-coding region involved in replication of the mtDNA molecule. The D-loop can range in size from 200 to greater than 1000 basepairs (Attardi 1985), and because it is not under the same constraints as protein coding regions, the majority of the D-loop is subject to both single base changes and length mutations. There are short regions of conserved sequences scattered throughout the D-loop which are necessary for the formation of secondary structures needed during replication (Brown et al. 1986, Southern et al. 1988, Hoelzel et al. 1991). Because there are no conserved regions at the light-strand 5' end of the D-loop, this region is considered highly variable and is often used in phylogenetic studies (Brown et al. 1993, Di Rienzo & Wilson 1991, Hoelzel 1991, Hoelzel & Dover 1991a, 1991b, Horai &

Hayasaka 1990, Rosel 1992, Shields & Kocher 1991, Stewart & Baker 1994, Thomas et al. 1990, Vigilant et al. 1989, 1991, Wilkinson & Chapman 1991). Estimates for the rate of evolution for the whole mitochondrial genome range from 0.5 - 2.0% change per million years (Myr) in primates to 0.6 - 1.8% change per Myr in *Drosophila* (Moritz et al. 1987, Wilson et al. 1985). In humans, the D-loop appears to evolve at 4-5 times the rate of the entire mitochondrial genome (Cann et al. 1987). Estimates for the rates of D-loop sequence divergence in mammals are as high as 10-20%/Myr in shrews (Stewart & Baker 1994), 8-17%/Myr in humans (Cann et al. 1989, Vigilant et al. 1989, 1991) and greater than 3.5%/Myr in kangaroo rats (Thomas et al. 1990).

Hoelzel et al. (1991) estimated the rate of divergence of cetacean D-loops from sequences they determined for the killer whale (*Orcinus orca*) and the minke whale (*Balaenoptera acutorostrata*), and from the previously published sequence for the Commerson's dolphin (*Cephalorhynchus commersonii*)(Southern et al. 1988). Their results confirmed that the outer regions of the D-loop are more variable in cetaceans than the central region but found that the rate of D-loop evolution in cetaceans is lower than that seen in other mammals. Assuming that mysticetes and odontocetes diverged 30-40 million years ago (minke whales versus killer whales and Commerson's dolphins) and that divergence within Delphinidae occurred within the last 5-10 million years (killer whales versus Commerson's dolphins)(Gaskin 1982), they estimated that the rate of D-loop evolution in cetaceans is 0.5%/Myr for the entire D-loop, with the rate outside the central region being 0.7%/Myr.

The entire D-loop sequence has also been determined for the sperm whale (*Physeter macrocephalus*)(Dillon & Wright 1993). They found that the D-loop

sequence of the sperm whale is longer than sequences from other cetacean species. In addition, they discovered that many of the conserved sequence blocks previously described in cetaceans (Hoelzel et al. 1991, Southern et al. 1988) are not present in the sperm whale, suggesting that the sequence blocks may not be functionally important.

The Cytochrome *b* Gene

The cytochrome *b* gene codes for a protein involved in the electron transport chain, and the structure-function relationships within the cyt *b* gene have been modelled (Howell 1989). Mutations within protein genes are usually constrained to silent nucleotide substitutions which do not result in amino acid changes (Thomas and Beckenbach 1989). Furthermore, spatial patterns of nucleotide substitution are affected by the structural and functional properties of regions within the protein (Kocher et al. 1989, Irwin et el. 1991). Despite these constraints, the cyt *b* gene can be used in population studies since it has been estimated that the cyt *b* gene has a silent divergence rate of approximately 5%/Myr for transitions (C↔T and A↔G) and 0.5%/Myr for transversions (all other base substitutions) at the third codon position for divergences up to 10 million years (Irwin et al. 1991).

RELEVANT STUDIES USING MITOCHONDRIAL DNA ANALYSIS

Sequencing and Phylogenetic Relationships of Cetacean Species

The entire mitochondrial genome has been sequenced from eight mammalian species, including two cetacean species (Anderson et al. 1981-human, Anderson et al. 1982-cow, Árnason et al. 1991-fin whale, Árnason et al. 1993-grey seal, Árnason & Gullberg 1993-blue whale, Árnason & Johnsson 1992-harbour seal, Bibb et al. 1981-mouse, Gadaleta et al. 1989-rat). The complete mtDNA sequences from the fin whale (*Balaenoptera physalus*) and the blue whale (*B. musculus*) were compared to investigate the amount of mitochondrial sequence variability between two species that hybridize in nature (Árnason & Gullberg 1993). The difference between the complete sequences was 7.4%, and they estimated that these species have been separated for at least 5 million years.

Mitochondrial ribosomal DNA sequences have been used to reexamine cetacean phylogeny (Milinkovitch et al. 1993). They determined that the sampled cetacean families, including dolphins (Delphinidae), porpoises (Phocoenidae), beaked whales (Ziphiidae), belugas and narwhals (Monodontidae) sperm whales (Physeteridae), and baleen whales (Balaenopteridae) were monophyletic. The phylogenetic analysis also indicated that the sperm whale may be more closely related to baleen whales (mysticetes) than other toothed whales (odontocetes). The Physeteridae were clustered with Balaenopteridae, and this cluster was consistently distinct from the cluster which included the Delphinidae, Phocoenidae, and Monodontidae. Only the placement of the Ziphiidae was uncertain after bootstrap

analysis. This result is surprising when compared to morphological evidence which has argued that mysticetes and odontocetes are distinct suborders (summarized in Gaskin 1982). However, comparative chromosome studies have indicated that sperm whales have karyotypes which are unique among the cetaceans and that they may have diverged very early in the evolution of cetaceans (Árnason 1974), and analysis of satellite DNA specific to cetaceans has suggested that sperm whales are more closely related to baleen whales than dolphins and porpoises (Grétarsdóttir & Árnason 1993).

Studies on Intraspecific and Interspecific Variation in Cetacean Species

During recent years, there has been an burgeoning of mtDNA research on cetacean species. Studies have been conducted using restriction enzyme analyses of whole mtDNA and PCR products, sequencing of cloned PCR products, and direct sequencing of PCR products. Studies of whole mtDNA restriction fragments have been done on humpback whales (*Megaptera noveangliae*) (Baker et al. 1990), beluga whales (*Delphinapteras leucas*) (Brennin 1992), minke whales (*Balaenoptera acutorostrata*) (Wada et al. 1991), bottlenose dolphins (*Tursiops truncatus*) (Dowling & Brown 1993, Duffield & Wells 1991), killer whales (Hoelzel 1991, Stevens et al. 1989), northern and southern right whales (*Eubalaena glacialis* and *E. australis*) (Schaeff et al. 1991), and spinner and spotted dolphins (*Stenella longirostris* and *S. attenuata*) (Dizon et al. 1991). Restriction enzyme analysis of PCR products has been used to study minke whales (*Balaenoptera acutorostrata*) (Hoelzel & Dover 1991b). Sequences from cloned PCR products have been used to enhance studies on minke whales

TABLE 1 : Summary of studies on intraspecific and interspecific mtDNA variation in cetacean species - columns refer to 1) the method used for analysis, 2) the species analyzed, 3) the number of individuals sampled, 4) comparisons between congeneric species, 5) comparisons between geographically distinct populations within a species, 6) comparisons between behaviorally defined groups within a species or population, 7) comparisons between morphologically distinct types within a species or population, and 8) the literature citation - in columns 4 through 7, "yes" indicates that the groups could be distinguished, "no" indicates that the groups could not be distinguished, and N/A indicates that the comparisons were not applicable to the study

METHOD	SPECIES	# INDIVS.	SPECIES?	GEOGR?	BEHAV?	MORPH?	CITATION
whole mtDNA							
RFLP-18 enzymes	humpback whales	84	N/A	yes	yes	N/A	Baker et al. 1990
RFLP-13 enzymes	minke whales	141	N/A	yes	N/A	yes	Wada et al. 1991
RFLP-10 enzymes	right whales	20	yes	N/A	N/A	N/A	Schaeff et al. 1991
RFLP-11 enzymes	belugas	71	N/A	yes	yes	N/A	Brennin 1992
RFLP-6 enzymes	killer whales	20	N/A	yes	yes	N/A	Stevens et al. 1989
RFLP-4 enzymes	killer whales	10	N/A	yes	yes	N/A	Hoelzel 1991
RFLP-1 enzyme	bottlenose dolphins	75	N/A	yes	yes	N/A	Duffield & Wells 1991
RFLP-6 or 8 enzymes	bottlenose dolphins	52	N/A	yes	yes	N/A	Dowling & Brown 1993
RFLP-11 enzymes	spinner dolphins	143	yes	yes	N/A	no	Dizon et al. 1991
D-loop							
RFLP-3 enzymes	minke whales	61	N/A	yes	N/A	N/A	Hoelzel & Dover 1991b
sequencing-560-bp	minke whales	3	N/A	yes	N/A	N/A	Hoelzel & Dover 1991b
sequencing-all or 362-bp	killer whales	9	N/A	yes	yes	N/A	Hoelzel & Dover 1991a
sequencing-404-bp	common dolphins	29	N/A	yes	N/A	yes	Rosel 1992
sequencing-394-bp	harbour porpoises	88	N/A	no*	N/A	N/A	Rosel 1992
sequencing-394-bp	porpoises	30	yes	yes	N/A	N/A	Rosel 1992
sequencing-400-bp	pilot whales	72	yes	no*	no	N/A	this study
cyt *b* gene							
sequencing-577-bp	harbour porpoises	30	yes	no*	N/A	N/A	Rosel 1992
sequencing-303-bp	pilot whales	22	yes	no*	no	N/A	this study

* - this refers to comparisons within ocean basins - in all four cases, differences were seen between ocean basins

(Hoelzel & Dover 1991b) and killer whales (Hoelzel 1991, Hoelzel & Dover 1991a), and direct sequencing of PCR products has been used to study harbour porpoises (*Phocoena phocoena*) and common dolphins (*Delphinus delphis*) (Rosel 1992). For a summary of these studies and chapter 2 of my dissertation, see Table 1.

Baker et al. (1990) surveyed whole mtDNA from 84 humpback whales sampled in two North Pacific feeding grounds, a Pacific winter breeding ground, and a North Atlantic feeding ground. Skin samples were collected using biopsy darts in southeastern Alaska (20 whales), central California (20 whales), Hawaii (16 whales), and the Gulf of Maine (28 whales). Twelve mtDNA haplotypes which differed at 1 - 7 sites were identified using 18 restriction enzymes. Nucleotide diversity was calculated among all individuals and within each population, and genetic distances were estimated between populations. The central California, Hawaii, and Gulf of Maine populations each contained more than one haplotype and had nucleotide diversity values which ranged from 0.028% to 0.196%. The Gulf of Maine haplotypes were distinct from the haplotypes found in the Pacific sites, and the genetic distance between the Gulf of Maine and Pacific sites ranged from 0.112% to 0.282%. The single southeastern Alaska haplotype was also the most common haplotype in the Hawaii population, and genetic distance values indicated that the southeastern Alaska and Hawaii populations were identical. Observations of marked individuals have shown that humpbacks from southeastern Alaska breed primarily in Hawaii, while humpbacks from central California breed primarily in Baja, Mexico, and maximum parsimony analysis of restriction site changes demonstrated that the geographical

distribution of Pacific haplotypes could be explained by these expected humpback migratory patterns.

The minke whale is probably the most abundant baleen whale. Because of this, the IWC whaling moratorium on this species has been challenged by a number of nations, and information about minke whale stock structure has become increasingly important. Wada et al. (1991) examined mtDNA from 141 minke whales sampled from the Sea of Japan (32 whales), the western North Pacific (30 whales), and Antarctic IWC management Areas IV (west of Australia) (40 whales) and V (east of Australia) (39 whales), and one dwarf minke whale from Area IV. They found 19 haplotypes using 13 restriction enzymes, and there were no shared haplotypes between the North Pacific whales, the Antarctic whales, and the dwarf minke whale. The average genetic distance within the minke populations ranged from 0% to 0.16%, although each population included two or more haplotypes, and G-tests showed that there were no significant differences in haplotype frequencies between the whales from the two North Pacific sites and between the whales from the two Antarctic sites. The genetic distance between populations ranged from 1.45% for the dwarf and North Pacific whales to 3.84% for the dwarf and Antarctic whales to 3.92% between the North Pacific and Antarctic whales. Consequently, they concluded that Antarctic minke whales should be distinguished as a separate species.

In another study on minke whales, Hoelzel and Dover (1991b) investigated genetic variation in 61 minke whales sampled from IWC Antarctic management Areas IV (21 whales) and V (20 whales), west Greenland (10 whales), and the western North Pacific (10 whales) using an amplified PCR product from mtDNA. The D-

loop, a non-coding region, was analyzed using 3 restriction enzymes, and 8 haplotypes were found. Each population had 3 or more haplotypes, and because the genetic distance between the two Antarctic populations was only 0.3%, these samples were combined. The average genetic distances within the three populations were all approximately 0.3%. The genetic distances calculated between populations were 3.7% for the Antarctic and west Greenland populations, 5.4% for the Antarctic and North Pacific populations, and 9.3% for the west Greenland and North Pacific populations. A 560-bp subclone of the D-loop was sequenced for one whale from Area V, the western North Pacific, and west Greenland. The Area V and North Pacific sequences differed by 19 base substitutions and 1 insertion, the Area V and Greenland sequences differed by 19 base substitutions and 2 insertions, and the Greenland and North Pacific samples differed by 9 base substitutions and 1 insertion. Interestingly, if genetic distances had been calculated using the sequence data, the resulting values would have been in reverse order to those calculated using the restriction enzyme data, indicating that these genetic distance estimates were sensitive to the molecular technique used and/or the sample size. However, sequencing and restriction enzyme analysis indicated that on a broad geographic scale, minke whale populations can be viewed as genetically distinct stocks.

Schaeff et al. (1991) surveyed whole mtDNA from 10 northern and 10 southern right whales in order to provide evidence that northern and southern right whales are separate species. Using 10 restriction enzymes, they found 7 mtDNA haplotypes, and there were no shared haplotypes between the two proposed right whale species. The northern right whales had 3 haplotypes, and the nucleotide

diversity within this species was 0.08%. The southern right whales had 4 haplotypes, and the nucleotide diversity within this species was 0.24%. The genetic distance calculated between the two species was 1.82%. Because the genetic distance between the northern and southern right whales was much greater than the average genetic distance within these groups and because the two proposed species shared no haplotypes, the results supported the hypothesis that northern and southern right whales are distinct species.

Brennin (1992) examined mtDNA variation in 71 belugas sampled from ten locations across North America and Greenland. Using 11 restriction enzymes, she identified 9 haplotypes, and sequence divergence among all of the belugas was 2.04%. The haplotypes were clustered into two major lineages. The first lineage was found primarily in the St. Lawrence Estuary and eastern Hudson Bay whales, while the second lineage was found primarily in the western Hudson Bay whales and whales from locations north of Canada and Alaska or west of Greenland. She hypothesized that the two lineages may exist because belugas have descended from two stocks that recolonized the Arctic after deglaciation 8000-9000 years ago. In addition, sequence divergence between the eastern and western Hudson Bay populations was 3.15%, even though whales from these two summering grounds seem to overwinter together in Hudson Strait. She attributed this divergence to maternally directed philopatry, and suggested that these two groups should be managed separately because depleted areas would not be recolonized by whales from other locations. However, because it is likely that the two groups of whales interbreed, she did not conclude that these groups were distinct genetic stocks.

Hoelzel (1991) mapped the mtDNA of 10 killer whales from the straits around Vancouver Island, British Columbia, and from Icelandic waters. The population of killer whales which inhabit waters around Vancouver Island has been intensively studied and is made up of 3 communities: coastal northern and southern resident communities, which consist of stable groups of pods and a sympatric transient community whose range includes the waters off Vancouver Island (Bigg 1982). Using 4 restriction enzymes, he found one HindIII site which was unique to Pacific killer whales and one EcoRI site which was unique to the transient killer whales around Vancouver Island. He then amplified the entire D-loop from one northern resident, one southern resident, and one transient whale from the communities off Vancouver Island. The northern and southern resident sequences differed by 1 base substitution and 1 deletion, the northern resident and transient sequences differed by 8 base substitutions and 1 deletion, and the southern resident and transient sequences differed by 7 base substitutions and 2 deletions. A HindIII/BamHI fragment (362-bp) which included the most variable portion of the D-loop was then subcloned and sequenced from one whale from Iceland, Germany, and Argentina, and from one additional individual from the three communities off Vancouver Island. The sequences of the two individuals from the three Vancouver Island communities were identical in this region. Genetic distance values were calculated between all of the populations sampled using data from the HindIII/BamHI fragment. The values ranged from 0% (Germany and transient populations) to 1.38% (Argentina and the transient or Iceland populations), and the genetic distance between the resident and transient communities using the HindIII/BamHI fragment was 0.55% (Hoelzel &

Dover 1991a). In addition, the genetic distances between the Vancouver Island communities were estimated from the entire D-loop sequences. The genetic distance between the two resident communities was 0.22% and the genetic distance between the resident and transient communities was 0.98%. The genetic distances between the Vancouver Island communities were comparable to the genetic distances between widely separated populations, indicating that the social structure of killer whale communities could be a significant factor influencing the mitochondrial genetic differentiation of killer whale populations. Because the composition of pods is stable, female reproductive dispersal and therefore mitochondrial gene flow between communities would be limited.

In another study which focused on Vancouver Island killer whales, Stevens et al. (1989) analyzed whole mtDNA from 20 captive killer whales. Ten of the whales were captured along the southeastern coast of Iceland, one of the whales was captive-born offspring of an Iceland female, four came from the northern resident community off Vancouver Island, one came from the southern resident community off Vancouver Island, two were captured from the transient community off Vancouver Island, and two stranded along the coast of Oregon. MtDNA was digested with 6 restriction enzymes, but only one enzyme, HaeIII, produced distinct patterns for the different killer whale populations. Three HaeIII patterns were produced, one which was found in the Atlantic whales, one which was found in the resident whales, and one which was found in the transient and stranded whales. The results of this study support the conclusions in Hoelzel (1991a).

Duffield and Wells (1991) began a preliminary analysis of whole mtDNA from 75 bottlenose dolphins as part of a comprehensive genetic study on two dolphin communities along the western coast of central Florida which have been part of a 20 year long-term observational study. Using only one restriction enzyme, HinfI, they found 4 haplotypes. Two of the haplotypes were found in the Sarasota Bay and Tampa Bay communities, and each of the two communities also had a unique haplotype. Consequently, they suggested that although the communities are based on distinct female matrilines, some females move between communities.

In another study of mitochondrial genetic variation in bottlenose dolphins, Dowling and Brown (1993) examined 52 dolphins from the Atlantic and Pacific. Using eight restriction enzymes, they analyzed whole mtDNA from 11 Pacific dolphins from Australian waters and 6 Atlantic dolphins, including 3 from the Gulf of Mexico and 3 from the eastern coast of Florida and North Carolina. The average pairwise sequence divergence between Atlantic and Pacific dolphins was 2.4%. Seven of the eleven Pacific dolphins had unique haplotypes, and pairwise divergence of haplotypes within the Pacific ranged from 0.08 to 0.58%. The Gulf of Mexico dolphins were distinct from the other Atlantic dolphins. Using six restriction enzymes, they also analyzed mtDNA from an additional 35 dolphins, including 28 Atlantic dolphins from the Gulf of Mexico, 5 Atlantic dolphins from the eastern coast of Florida, and 2 Pacific dolphins from the Gulf of California. Among these samples, the average pairwise sequence divergence between Atlantic and Pacific dolphins was 1.5%. Five haplotypes were found among the 28 Gulf dolphins, and the heterozygosity was 0.61. The other Atlantic dolphins had the same haplotype, and it

was distinct from the Gulf haplotypes. They concluded that although gene flow within the Gulf of Mexico is considerable, gene flow does not occur between the Gulf and Atlantic coasts of Florida. They suggest that this dichotomy may exist because dolphins from the two coasts of Florida forage on organisms that are geographically separated by ocean currents.

Dizon et al. (1991) examined whole mtDNA from spinner dolphins in the eastern tropical Pacific (ETP). Spinner dolphins are frequently killed incidentally by the tuna purse seine fishery, and the ETP population is divided into 2 management units based on the morphology of the dolphins, a coastal eastern form and a more cosmopolitan whitebelly form. Seventy-nine eastern spinner, 45 whitebelly spinner, and 8 spotted dolphins from the ETP and 11 spinner dolphins from the Timor Sea were screened using 11 restriction enzymes, and 55 unique haplotypes were found. Each of the 4 sampled groups contained 3 or more haplotypes, and the average genetic distance within groups ranged from 0.3% (Timor Sea) to 1.7% (spotted dolphins). The genetic distances between the 3 spinner dolphin groups ranged from 0.9% - 1.4%, and the average genetic distance between the spinner dolphins and the spotted dolphins was 4.4%. They were able to identify haplotypes which were found only in the spotted dolphins and the Timor Sea spinner dolphins. However, within the ETP, they found only weak correspondence between mtDNA haplotypes and morphology, and they hypothesized that the localized eastern form was being lost due to hybridization with the more common and less endemic whitebelly form.

Rosel (1992) undertook a study on the common dolphin, another species taken in the ETP tuna purse seine fishery. This species is currently divided into

management units based on latitude and morphology: northern, central, and southern short-beaked forms, and a long-beaked form which is sympatric with the northern short-beaked form. She amplified and sequenced a 404-bp fragment from the light-strand 5'-end of the D-loop from 11 long-beaked dolphins, 8 northern short-beaked common dolphins taken in the coastal waters off California, 6 central short-beaked dolphins from the ETP, and 4 short-beaked dolphins from the Black Sea. Nucleotide diversity for all of the dolphins was 2.10%, for the short-beaked dolphins was 1.80%, and for the long-beaked dolphins was 1.60%. Although some dolphins within morphotypes had completely identical D-loop sequences, no complete D-loop sequences were shared by dolphins of different morphotypes, and there was a fixed nucleotide difference between the two morphotypes. DNA divergence between the short-beaked and long-beaked dolphins was 1.85%, and the divergence of 1.78% between the sympatric northern short-beaked and long-beaked dolphins was greater than the divergence between the geographically separated California and ETP plus Black Sea short-beaked dolphins. Consequently, she suggested that the long-beaked common dolphin should be classified as a distinct subspecies.

The harbour porpoise is taken incidentally by a number of fisheries, and in many areas, the populations may be seriously threatened by this loss. In order to investigate harbour porpoise stock structure, Rosel (1992) examined harbour porpoises from the eastern North Pacific (64 porpoises), the western North Atlantic (7 porpoises), the eastern North Atlantic (8 porpoises), and the Black Sea (9 porpoises). She amplified and sequenced a fragment which included 394-bp from the light-strand 5'-end of the D-loop and 112-bp from transfer RNAs. Nucleotide

diversity for all of the porpoises was 1.6%, and ranged from 0.7% for the North Pacific porpoises, 0.4% for the North Atlantic, and 0.05% for Black Sea porpoises. DNA divergence between oceans was 5.5% for the North Pacific versus North Atlantic porpoises, 5.8% for the North Pacific versus Black Sea porpoises, and 2.2% for the North Atlantic versus Black Sea porpoises. The porpoises sampled in the North Pacific came from California, Washington, British Columbia, and Alaska, and the mtDNA sequences she determined could be clustered into two major groups. However, despite the fact that harbour porpoises are coastal animals which do not appear to migrate the length of the entire North American coastline, she was unable to find any relationship between mtDNA sequences and geographic locations. This unexpected result caused her to hypothesize that eastern North Pacific harbour porpoise population may include two sympatric but distinct breeding stocks or two historically isolated stocks that have undergone secondary contact.

Rosel (1992) also used mtDNA sequences to examine the phylogenetic relationships of species in the porpoise family (Phocoenidae). She sequenced a 526-bp fragment from the light-strand 5'-end of the D-loop and transfer RNAs and a 577-bp fragment from the cyt *b* gene from 10 eastern North Pacific harbour porpoises, 9 Black Sea harbour porpoises, 7 North Pacific Dall's porpoises (*P. dalli*), 15 vaquitas (*P. sinus*), 6 Peruvian Burmeister's porpoises (*P. spinipinnis*), 4 Argentinean spectacled porpoises (*P. dioptrica*), and 2 Chinese finless porpoises (*Neophocoena phocaenoides*). Overall, she found that there was a 64% A-T bias in the sequences and that the 5'-end of the D-loop fragment was highly divergent compared to the 3'-end which included the conserved blocks reported in other cetacean species (Hoelzel

et al. 1991, Southern et al. 1991). Although the phylogenies based on the D-loop and cyt *b* gene sequences were not completely congruent, all of the porpoises fell out in a group which was distinct from the dolphin species she used as outgroups. Sequence variability in the eastern North Pacific harbour and Dall's porpoises was high, but little or no variation was found among the Black Sea harbour porpoises or the vaquitas. Because both populations were isolated during the Pleistocene glacial periods (15,000 years ago), she hypothesized that they may have gone through a bottleneck.

The measures of genetic variability used in the studies described above were not consistent, and the methods used for analyses varied between studies. Consequently, it is not possible to compare all of the studies on the basis of their numerical results. However, a number of important generalizations can be made (Table 1). These studies found that patterns of genetic variation in cetaceans can be correlated with known or suspected patterns of geographic separation in most of the species studied. One exception was the harbour porpoise, where no relationship was found between the D-loop sequences and geographic sampling locations of eastern North Pacific harbour porpoises, although Pacific and Atlantic porpoises could be easily distinguished (Rosel 1992). In addition, known patterns of association and behavior, such as shared wintering and summer feeding grounds in humpbacks (Baker et al. 1990), summering grounds in belugas (Brennin 1992), stable social groups in killer whales (Hoelzel 1991, Hoelzel & Dover 1991a, Stevens et al. 1989), and female matrilines and foraging behavior in bottlenose dolphins (Dowling & Brown 1993, Duffield & Wells 1991) can significantly affect patterns of genetic variation. The

relationship between morphology and genetic variation is less predictable. In spinner dolphins, there was only weak correspondence between morphologically defined management units and mtDNA haplotypes distinguished using restriction enzymes (Dizon et al. 1991). However, in common dolphins, the long-beaked and short-beaked forms did not share any D-loop sequences and analysis of the DNA divergence between sympatric long-beaked and short-beaked dolphins versus geographically separated short-beaked dolphins suggested that the two morphotypes might be distinct subspecies (Rosel 1992). In addition, in minke whales, the genetic distance between the dwarf-form and other sympatric Antarctic minke whales was greater than the genetic distance between the dwarf-form and morphologically similar North Pacific minke whales (Wada et al. 1991).

The D-loop

D-loop sequence variability has been used in a number of population genetic studies. In addition to those described above on cetaceans, studies have been done on humans (Di Rienzo & Wilson 1991, Horai & Hayasaka 1991, Vigilant et al. 1989, 1991), kangaroo rats (Thomas et al. 1990), bats (Wilkinson et al. 1991), rabbits (Biju-Duval et al. 1991), monkeys (Hayasaka et al. 1991), and shrews (Stewart & Baker 1994). The studies and chapter 2 of my dissertation are summarized in Table 2.

Sequences from two highly variable regions at either end of the D-loop totaling 740-bp have been used to study human populations. Vigilant et al. (1989) determined D-loop sequences for 83 humans, including 15 !Kung, 17 western Pygmies, 20 eastern Pygmies, and 31 other individuals from Asia, Africa, Europe, and

TABLE 2 : Summary of studies on D-loop variation in mammalian species - columns refer to 1) the species analyzed, 2) the number of individuals sampled, 3) the length of the sequenced D-loop fragment, 4) the number of observed mtDNA haplotypes, 5) nucleotide diversity among all of the sampled individuals, 6) average pairwise sequence divergence between all of the sampled individuals, 7) presence or absence of heteroplasmy due to tandem repeat length polymorphisms in the D-loop, and 8) the literature citation - in column 3, N/A indicates that the D-loop was not sequenced, and in columns 4 through 6, N/A indicates that this measure of diversity for the D-loop was not given in or easily calculated from data in the cited paper. The sequenced D-loop fragment and the tandemly repeated length polymorphisms which caused heteroplasmy were located at the light-strand 5'-end of the D-loop unless otherwise indicated.

SPECIES	# INDIVS.	LENGTH	# TYPES	NUC. DIV.	SEQ. DIV.	HETERO?	CITATION
long-finned pilot whales	59	400-bp	3	0.03%	0.06%	no	this study
short-finned pilot whales	13	400-bp	3	0.05%	0.11%	no	this study
bottlenose dolphins	11	400-bp	11	1.25%	2.50%	no	this study
common dolphins	29	404-bp	27	2.10%	N/A	no	Rosel 1992
harbour porpoises	88	394-bp	33	1.6%	N/A	no	Rosel 1992
minke whales	61	N/A	8*	N/A	N/A	no	Hoelzel & Dover 1991b
minke whales	3	560-bp	3	1.49%	3.04%	no	Hoelzel & Dover 1991b
killer whales	9	362-bp	6	0.25%	0.51%	no	Hoelzel & Dover 1991a
killer whales	3	921-bp	3	0.35%	0.72%	no	Hoelzel 1991
humans	189	610-bp	135	N/A	1.08-2.08%**	no	Vigilant et al. 1991
humans	101	482-bp	94	1.45%	N/A	no	Horai & Hayasaka 1990
humans	117	620-bp	88	N/A	N/A	no	DiRienzo & Wilson 1991
kangaroo rats	106	225-bp	24	N/A	2.20%	no	Thomas et al. 1990
evening bats	195	350-bp	N/A	N/A	N/A	yes	Wilkinson & Chapman 1991
European rabbits	121	N/A	N/A	N/A	N/A	yes***	Biju-Duval et al. 1991
Japanese monkeys	100****	650 or 810-bp*****	N/A	N/A	N/A	yes***	Hayasaka et al. 1991
masked shrews	19	700-900-bp	19	N/A	N/A	yes	Stewart & Baker 1994

* - 8 whole D-loop haplotypes were identified using 3 restriction enzymes
** - these average sequence divergences were calculated within human race groups of 15-24 individuals
*** - heteroplasmy was due to tandem length repeats at the light-strand 3'-end of the D-loop
**** - D-loop sequences were determined from only 4 monkeys - other monkeys were analyzed based on length polymorphisms and presence of restriction sites
***** - the sequenced fragment was located at the light-strand 3'-end of the D-loop

the United States. Within the 15 !Kung sampled, there were one pair and one quartet of individuals who had identical sequences. Within the 17 western Pygmies sampled, there were one pair and one trio of individuals who had identical sequences, and within the eastern Pygmies sampled, there were three pairs and one trio of individuals who had identical sequences. They calculated that the probability that two individuals would have identical sequences was 0.13 for the !Kung, 0.09 for the western Pygmies, and 0.08 for the eastern Pygmies. Estimates of gene diversity for the three groups ranged from 0.93 for the !Kung to 0.97 for the two Pygmy groups, indicating that mtDNA diversity in the !Kung population was relatively low. In the later study, Vigilant et al. (1991) expanded their sample size to 189 individuals. The sample included 121 native Africans (25 !Kung, 27 Herero, 1 Naron, 17 Hadza, 14 Yorubans, 20 Eastern Pygmies, and 17 Western Pygmies) and 68 other humans (20 Papua New Guineans, 1 Australian, 15 Europeans, 24 Asians, and 8 African Americans). They sequenced 610-bp from each individual, and identified 135 different D-loop sequences among the 189 individuals. Individuals with identical sequences were found only within sampled populations. The average pairwise sequence divergence among Africans (2.08%) was greater than the divergence among Asians (1.75%) or Caucasians (1.08%). Because older populations have more time to accumulate mutations, these divergences suggested that the common human ancestor was of African origin. This was further confirmed with a parsimony tree built using the chimpanzee as an outgroup, where the first 14 mtDNA types that diverged from the tree were African. Using the corrected pairwise sequence divergence between human and chimpanzee mtDNA and an estimate that humans

and chimpanzees diverged 4-6 million years ago, they estimated that the rate of divergence of the outer regions in the D-loop was 11.5%-17.3%/Myr. Consequently, they estimated that the common human ancestor lived 140,000 to 249,000 years ago.

Horai and Hayasaka (1991) also undertook a study of D-loop sequence variation among 95 humans of African, Asian, and Caucasian origin, and their results were similar to those of Vigilant et al. (1991). They cloned and sequenced 482-bp at the light-strand 5'-end of the D-loop from 61 Japanese, 3 Koreans, 4 Chinese, 1 Indonesian, 1 Filipino, 1 Papua New Guinean, 17 Caucasians from Europe, North America, and India, and 7 Africans. The nucleotide diversity among all of the individuals was 1.45%, and diversity among the Africans was greater than among the other groups. Distance trees built using cluster analysis and neighbor-joining methods divided Asians into two distinct groups and showed that the first mtDNA types to diverge were of African or Asian origin.

Di Rienzo and Wilson (1991) examined mitochondrial D-loop sequence variation in humans as well. They sequenced a 620-bp fragment from the light-strand 5'-end of the D-loop from 69 Sardinians, 29 Bedouins, 8 Israeli Arabs, 5 Yemenite Jews, and 6 other Caucasian individuals. They found 88 distinct sequences, and all but two of the shared mtDNA types were found only in Sardinia or the Middle East. However, lineages from the Sardinian and Middle Eastern populations were scattered throughout the mtDNA parsimony tree, indicating that these populations are related. Because most of the branches originated at the 0.5-0.75% sequence divergence level and because the frequency distributions of sequence differences between all possible pairs in each population were approximately Poisson, they concluded that if the

mtDNA types were neutral, mtDNA lineage survival in Caucasian populations has changed during human evolution, possibly due to rapid population growth during the geographic expansion of humans outside of Africa.

In another study involving D-loop sequence variation, Thomas et al. (1990) examined 3 populations of kangaroo rats (*Dipodomys panamintinus*) using modern and museum specimens. Sequences from a total of 106 individuals were determined from PCR products which included part of the threonine tRNA, the proline tRNA, and the light-strand 5' end of the D-loop, and 19 of 225 basepairs were variable. Most of the sequence variation was present in the D-loop. The number of kangaroo rats examined from modern or museum specimens for the 3 populations ranged from 8 to 24 individuals. The average genetic distances within populations ranged from 0.22% to 1.72%, and gene diversities ranged from 0.22 to 0.89. Temporal samples for the 3 populations shared some mtDNA types, and levels of genetic diversity for temporal samples for each of the samples were similar. When corrected interpopulation and temporal genetic distances were used to construct a population tree, the modern and museum specimens from each population fell into the same clades. In addition, molecular trees based on informative sites between mtDNA types demonstrated that major mtDNA lineages present in the modern samples were also present in the museum specimens.

Heteroplasmy, defined as having more than one mtDNA type per individual or cell, has been seen frequently in invertebrates and poikilothermic vertebrates and was believed to be rare in mammals (Bermingham et al. 1986, Moritz et al. 1987). However, recent studies of D-loop sequence variation in cows, rabbits, bats, pigs,

monkeys, and shrews have revealed that heteroplasmy may be more common in mammals than previously thought (Biju-Duval et al. 1991, Ghivizzani et al. 1993, Hauswirth et al. 1984, Hayasaka et al. 1991, Mignotte et al. 1990, Stewart & Baker 1994, Wilkinson & Chapman 1991).

Wilkinson and Chapman (1991) analyzed D-loop sequence variation among 195 evening bats (*Nycticeius humeralis*) sampled from seven colonies and discovered that these bats can be heteroplasmic. They identified an 81-bp fragment that was tandemly repeated five to eight times at the light-strand 5'-end of the D-loop, and this resulted in length variants that were found within and between individuals. Heteroplasmy was seen in 28% of the bats, and in all but one case, heteroplasmic pups had heteroplasmic mothers with the same PCR band patterns. One heteroplasmic pup had a mother and a sibling which were homoplasmic. The frequency of heteroplasmy was not significantly different between colonies, although the frequencies of different mtDNA types did vary between colonies. Base substitutions did occur within repeats, and because adjacent repeats were more similar to each other than to more distant repeats, they concluded that the repeats were formed during replication slippage. They estimated that the mutation rate per generation for length polymorphisms is 10^{-2}. Since this rate is so rapid, they concluded that length repeat polymorphisms alone would not be useful for population studies because mutations would occur too rapidly to allow different repeat types to become fixed in a population. However, if the sequences of the repeats were analyzed as well, matrilines could be identified.

Biju-Duval et al. (1991) analyzed D-loop variation between 107 European rabbits (*Oryctolagus cuniculus*), 13 European hares (*Lepus capensis*), and 1 eastern European cottontail (*Sylvilagus floridanus*). Using 11 restriction enzymes, they identified 17 mtDNA haplotypes among the European rabbits, and these haplotypes were divided into two groups found in rabbits from the distinct geographic locations of southern Spain or northern Spain, France and Tunisia. In addition, they examined D-loop length repeat polymorphisms using two probes developed by Mignotte et al. (1990). These probes hybridize to two distinct stretches of tandem repeats of 20-bp or 153-bp at the light-strand 3'-end of the D-loop. Because all of the sampled animals were heteroplasmic, they concluded that heteroplasmy is a shared derived character in the rabbit family (Leporidae).

Hayasaka et al. (1991) examined D-loop variability in 100 Japanese monkeys (*Macaca fuscata*) sampled from 12 localities throughout Japan. They cloned and sequenced mtDNA from 4 monkeys and located a 160-bp tandem repeat at the light-strand 3'-end of the D-loop which was responsible for length polymorphisms. Using PCR, they amplified this part of the D-loop and identified eight mtDNA types on the basis of the length of the PCR product and the presence or absence of two restriction enzyme recognition sites. Four of the mtDNA types had only one copy of the 160-bp repeat, and one of these types was found in monkeys from seven of the 12 localities. The other four identified types always occurred together as fixed pairs in monkeys with heteroplasmic mtDNA, and in both cases, the mtDNA types in these pairs had two or three copies of the 160-bp repeat. Each of the heteroplasmic pairs of mtDNA

was limited to monkeys from one locality, and all of the monkeys sampled from each of these two localities were heteroplasmic for the identified pair of mtDNA types.

Stewart and Baker (1994) also investigated the utility of length polymorphisms in the D-loop for phylogenetic and population studies. They sequenced the light-strand 5'-end of the D-loop from 18 shrews belonging to the masked shrew group (*Sorex cinerius cinerius, S.c. miscix, S.c. adacicus,* and *S. haydeni*) and one pygmy shrew (*S. hoyi*). They discovered a region containing at least five 79-bp tandem repeats and a 76-bp imperfect repeat in each shrew, and one shrew was heteroplasmic for five and seven repeats. Additional length variation was found in a 163-bp variable region between the repeats and the proline tRNA, and this region varied in length by as much as 40-bp. They calculated pairwise sequence divergences between each of the masked shrew groups and the pygmy shrew for the fifth repeat, the imperfect repeat, and variable region. The variable region was the least conserved, and the imperfect repeat was the most conserved. The average pairwise sequence divergence for all of the regions ranged from 0.15±0.03 to 0.20±0.03, and based on this data, they concluded that the rate of D-loop sequence divergence in shrews is 10-20%/Myr. In addition, they also concluded that the mutation rate for length polymorphisms was much higher than the mutation rate for base substitutions, deletions, and insertions.

The Cytochrome *b* Gene

Cyt *b* gene sequences have been used to examine intraspecific genetic variation in a limited number of studies. Smith and Patton (1991) used sequences from a 401-bp fragment from the light-strand 5' end of the cyt *b* gene to study 12

species of akodontine rodents. They sampled a total of 20 populations, and when possible, cyt *b* sequences were determined for two individuals from each population. In 13 of the 20 populations, both individuals were identical, and in 6 of the populations, the sequences from the two individuals differed by 1 to 3 base substitutions. In a study on the evolution of the cyt *b* gene, Irwin et al. (1991) sequenced the entire cyt *b* gene (approximately 1140 bp) from one or more individuals belonging to 14 ungulate and 2 cetacean species, the spinner dolphin and the spotted dolphin, using direct sequencing of single-stranded PCR products and sequencing of cloned PCR products. Sequences were obtained for two captive spinner dolphins, and the sequences differed by 17 base substitutions, or roughly 1.5% of the entire cyt *b* sequence.

Patterns of Mitochondrial Genetic Variation Affected by Female Behavior

The studies on humpback whales, belugas, killer whales, and bottlenose dolphins described earlier indicate that female behavior can affect patterns of mtDNA variation. In sea turtles, the effect of female versus male behavior has been explicitly examined using mitochondrial and nuclear genetic markers. Tagging studies have shown that female green turtles return to their natal rookeries to nest, and if turtles are sampled at rookeries, females appear to not disperse. MtDNA analyses on green turtles revealed geographic patterns of genetic variation that correspond to rookeries (Bowen et al. 1992). Using 17 restriction enzymes, they found 8 mtDNA haplotypes in rookeries from the Atlantic Ocean/Mediterranean Sea and 6 haplotypes in rookeries from the Pacific/Indian Oceans, and none of the haplotypes were found

in both ocean basins. An estimator of interrookery gene flow (*Nm* from Slatkin 1989) determined that there was little gene flow between rookeries. However, restriction fragment analysis of nuclear DNA showed that common nuclear haplotypes were found in most rookeries throughout the world, suggesting that male-mediated gene flow does occur in this species (Karl et al. 1992).

CONCLUDING REMARKS

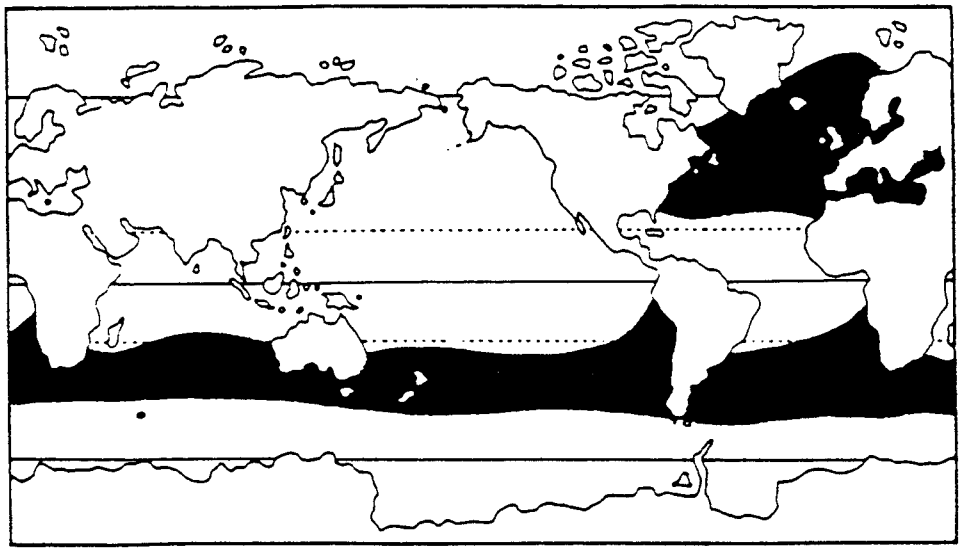
One goal of management policies should be to preserve adapted genetic variation. Lack of genetic variation within a population could endanger its survival because populations which do not possess genetic variants might not be able to adapt to changing environments. It is conceivable that extinction of a single genetic stock could result in the loss of important rare alleles necessary for the future survival of a species. Furthermore, if populations are to survive and be maximally productive, their conservation must include efforts to maintain sufficient numbers of individuals possessing sufficient levels of genetic variation to avoid the detrimental effects of inbreeding resulting from random genetic drift within a small population. It is frequently mentioned that information from genetic markers such as mtDNA is not reliable on its own for describing genetic stocks because these neutral genes are not affected by natural selection, and patterns of variation of neutral genetic markers might not reflect patterns due to adaptive evolution (Dizon et al. 1992, Lande 1991, Palumbi et al. 1991). For instance, genetic drift might cause mtDNA types to diverge between two populations that had been separated for a short time, even though the

populations did not have time to evolve under different environmental conditions and there was no net genetic divergence between them. Alternatively, morphologically distinct members of a species might not be distinguished using mtDNA because even limited exchange of mtDNA types during hybridization events is sufficient to prevent substantial mitochondrial genetic divergence between the two morphotypes (as in spinner dolphins - Dizon et al. 1991). Consequently, although information obtained using neutral genetic markers can be extremely valuable, it must be interpreted in conjunction with other sources of information such as abundance estimates, migration patterns, contaminant and parasite loads, fishery interactions, demographic variables, behavior, and morphology (Dizon et al. 1992, Ryder 1986). In particular, when mtDNA is used, female behavior can have a significant effect on patterns of genetic variation (as in sea turtles - Bowen et al. 1992).

The review of studies on cetaceans has indicated that mtDNA variation can be correlated with geographical or behavioral separation in most of the species studied, and could therefore provide initial rationale for defining genetic stocks.. However, because the reproductive dispersal behavior of female and male pilot whales differs, it is possible that patterns of mtDNA variation in pilot whales will not reflect their genetic stock structure in the North Atlantic.

FIGURE 1 : Distribution of *Globicephala* species (from Watson 1981) - The long-finned pilot whale, *G. melas*, has an anti-tropical distribution in temperate and sub-Arctic waters in the North Atlantic, South Atlantic, South Pacific, and Indian Oceans. The short-finned pilot whale, *G. macrorhynchus*, has a circumpolar distribution in tropical and warm temperate waters. Stranding and sighting records indicate that the ranges of the two species may overlap between 35°N and 40°N latitudes (IWC 1990, Sergeant 1982).

Globicephala melas



Globicephala macrorhynchus

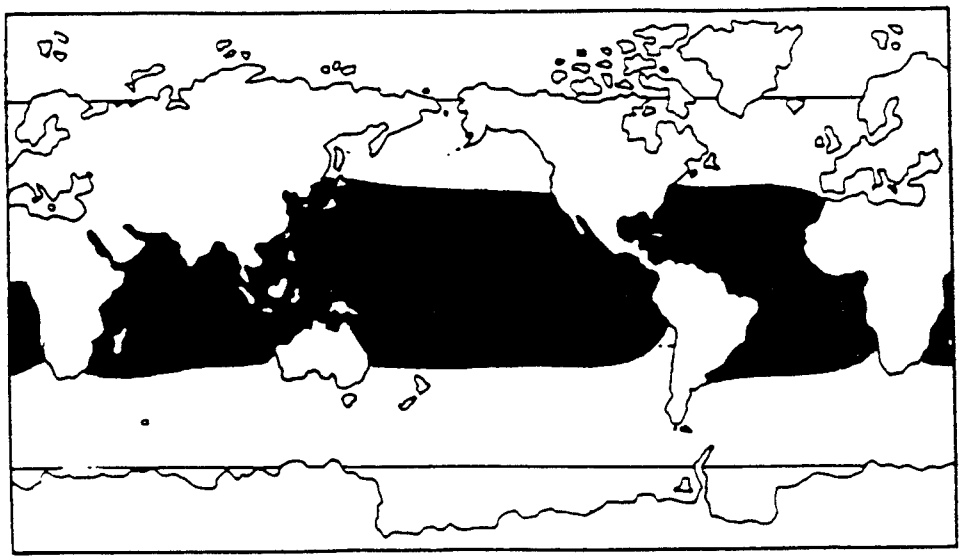
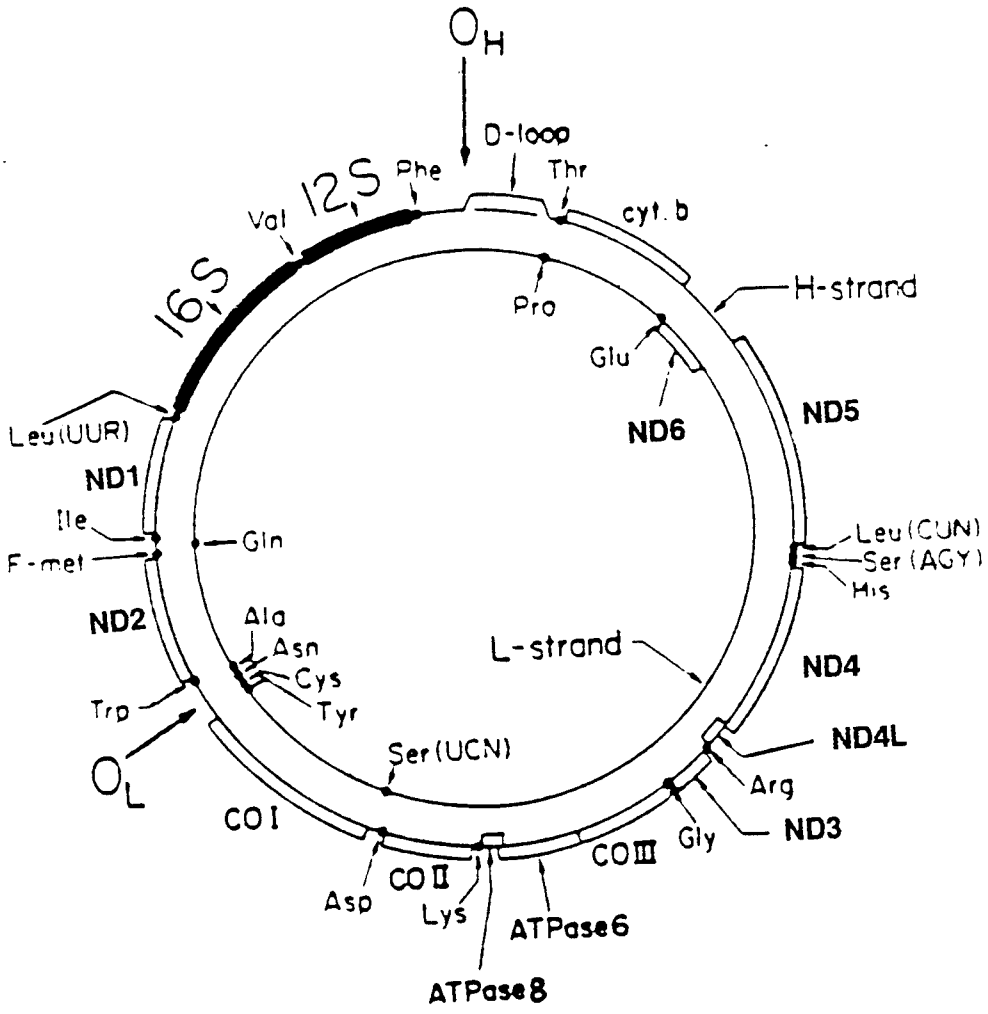


FIGURE 2 : Mammalian mitochondrial DNA (updated from Attardi 1985) - This map of mtDNA from a human cultured cell line shows the organization of mtDNA that is common to all mammals studied to date.



APPENDIX : Formulas used to estimate measures of genetic variation

1. Estimation of genetic distance

Although other symbols, such as D, p, and δ are often used to represent genetic distance, the symbol d will be used throughout this appendix. In addition, r will be used to designate the number of nucleotides in a restriction enzyme recognition site.

a) estimation of genetic distance from restriction fragments - Nei & Li 1979

$$d = - (2/r) \ln G$$

G can be estimated from F, the proportion of fragments shared by 2 haplotypes, using the formula $F = G^4/(3 - 2G)$

where $F = 2 n_{xy}/(n_x + n_y)$

and n_{xy} = the number of fragments shared by haplotypes x and y
n_x = the total number of fragments in haplotype x
n_y = the total number of fragments in haplotype y

b) estimation of genetic distance from restriction fragments - Upholt 1977

$$d = 1 - \{[-F + (F^2 + 8F)^{1/2}]/2\}^{1/r}$$

where $F = 2 n_{xy}/(n_x + n_y)$ as above

c) estimation of genetic distance from sequence data - Kimura & Ohta 1972, Nei 1987

$$d = - 3/4 \ln (1 - 4/3k)$$

where k = the percent difference in base composition between 2 sequences

This formula can be used when the four nucleotides are present in equal proportions, and all types of substitutions are equally probable.

If the four nucleotides are not present in equal proportions, the general formula below can be used (Hillis & Moritz 1991).

$$d = - b \ln (1 - 1/bk)$$

where $b = 1 - \Sigma f_i^2$

and f_i = the frequency of each nucleotide

In mtDNA, transitions (C↔T, G↔A) outnumber transversions (all other substitutions) by approximately 9 to 1, and the formula from Brown et al. (1982) can be used to correct for this bias

$$d = 1/2 \, [1/2(d_i + d_v) + (d_i n_i + d_v n_v)/(n_i + n_v)]$$

where n_i = the number sites which differ by a transition
n_v = the number of sites that differ by a transversion

and $d_i = - 5/9 \ln (1 - 2k_i)$
$d_v = - 20/3 \ln (1 - 3/2k_v)$

where k_i = the fraction of sites that differ by transitions
k_v = the fraction of sites that differ by transversions

2. Estimation of nucleotide diversity, π - Nei 1987

$$\pi = (n/n\text{-}1) \Sigma \, x_i x_j \pi_{ij}$$

where n = the number of individuals sampled from the population

and x_i and x_j = the estimated population frequency of the i^{th} and j^{th} type of DNA sequence

and π_{ij} = the proportion of different nucleotides between the i^{th} and j^{th} type of DNA sequence

3. Estimation of gene diversity, h - Nei 1987

$$h = n(1 - \Sigma x_i)/(n - 1)$$

where n = the number of individuals sampled from the population

and x_i = the estimated population frequency of the i^{th} allele (or sequence)

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CHAPTER TWO

Mitochondrial DNA Sequence Variation within the Genus *Globicephala*, with an Emphasis on the North Atlantic Long-finned Pilot Whale, *Globicephala melas*

INTRODUCTION

The long-finned pilot whale, *Globicephala melas*, inhabits temperate and subpolar waters throughout the North Atlantic. Pilot whales are incidentally taken by commercial fisheries operating in US waters (Douglas 1989, Waring et al. 1990), and are or have been hunted in parts of their range (Andersen 1988, Mitchell 1975a, 1975b, Sergeant 1962). Additional mortalities occur during mass strandings (Sergeant 1982). In order to assess the impact of these losses, it is important to understand the genetic structure of the North Atlantic population. If it is comprised of geographically separated and genetically differentiated subpopulations, local mortalities which threaten these subpopulations could result in the loss of genetic variation necessary for the future health and survival of the species.

In order to determine if there is more than one genetically differentiated subpopulation, or genetic stock, of long-finned pilot whales within the North Atlantic, I sequenced mitochondrial DNA (mtDNA) from 59 whales that stranded on the coasts of Cape Cod, Newfoundland, Nova Scotia, Scotland, and England or were incidentally caught by commercial fisheries operating in the western North Atlantic.

Samples from 11 stranded or incidentally caught Atlantic short-finned pilot whales, *Globicephala macrorhynchus*, and two incidentally caught Pacific short-finned pilot whales were analyzed to provide information about interspecific mitochondrial genetic variation in the genus *Globicephala*. In addition, 11 North Atlantic bottlenose dolphins, *Tursiops truncatus*, were also examined to assess mitochondrial genetic variation in another cetacean species taken in waters from which many of the sampled long-finned pilot whales originated. MtDNA sequences were obtained for the D-loop, a non-coding region, and from the protein gene coding for cytochrome *b*.

METHODS

Tissue samples were collected from stranded and incidentally caught long-finned pilot whales from 4 strandings on Cape Cod (n = 24), 16 incidental catches in the western North Atlantic (n = 24), one stranding on Sable Island, Nova Scotia (n = 3), one stranding in western Newfoundland (n = 1), one stranding in western Scotland (n = 4) and 2 strandings in England (n = 3). Atlantic short-finned pilot whale samples were collected from 4 strandings in Delaware and North Carolina (n = 4), and 4 incidental catches in the western North Atlantic (n = 7). Pacific short-finned pilot whale samples were collected from one incidental catch off California (n = 1) and one incidental catch in the eastern tropical Pacific (n = 1). Tissue samples from 11 bottlenose dolphins (*Tursiops truncatus*), collected during 10 incidental catches in the western North Atlantic, were also analyzed. Details about pilot whale

and bottlenose dolphin sample collection events, localities, and dates are listed in Tables 1, 2, and 3.

DNA Extractions

Total genomic DNA was isolated from small skin, heart, liver, muscle, kidney, or gonad samples weighing less than 100 mg using standard techniques. The samples were chopped into small pieces or crushed, and digested at 37-40°C for at least 2 hours in 1 ml of lysis buffer (1% Triton X-100 detergent, 4 M urea, 10 mM EDTA, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 10 mM DTT) with a fresh solution of proteinase K added to a final concentration of 0.8 mg/ml. Digested samples were extracted at least 2 times with equal volumes of phenol and once with an equal volume chloroform. DNA was precipitated from the final aqueous layer overnight at -20°C or for 1 hour at -70°C using 2 volumes of ethanol and 1/5 volume of 3 M sodium acetate or 5 M ammonium acetate. The resulting DNA pellets were washed twice with 70% ethanol, dried in a speed-vac, and resuspended in 30-100 *u*l of sterile 1xTE buffer. In most cases, a 1:10 or 1:100 dilution of the resuspended DNA was used for amplification reactions.

DNA Amplifications and Sequencing

Portions of the D-loop and the cyt *b* gene were amplified in a polymerase chain reaction (PCR) using a series of primers (Figure 1 and Table 4). A 350-bp fragment at the 5' end of the cyt *b* gene was amplified using two primers (cytbL26 and cytbH24) which were modified from universal primers described in Kocher et al.

TABLE 1 : Long-finned pilot whale samples - All available information is included in the table. A "?" is present wherever information about the date of sample acquisition is not included in source institution records.

	EVENT	# WHALES	DATE & LOCATION
	bycatch	1	? - western North Atlantic
	bycatch	1	? - western North Atlantic
	bycatch	1	?/?/89 - western North Atlantic
	bycatch	2	?/?/89 - western North Atlantic
	bycatch	1	?/?/89 - western North Atlantic
	bycatch	3	3/9/90 - 3932N/7246W
	bycatch	1	4/5/90 - 3900N/7300W
	bycatch	2	4/6/90 - 3954N/7157W
	bycatch	3	4/16/90 - 4014N/7055W
	bycatch	1	4/24/90 - 4010N/7001W
	bycatch	1	4/25/90 - 4009N/7001W
	bycatch	1	4/26/90 - 4010N/7001W
	bycatch	2	4/27/90 - 4007N/7019W
	bycatch	1	4/30/90 - 4009N/7001W
	bycatch	2	?/?/92 - western North Atlantic
	bycatch	1	?/?/92 - western North Atlantic
	stranding	13	12/11/90 - Hyannis, MA
	stranding	5	9/29-10/2/91 - Dennis/Sandwich/Truro, MA
	stranding	1	9/9-9/10/91 - Wellfleet/Brewster, MA
	stranding	5	9/24-9/25/91 - Eastham, MA
	stranding	3	7/29/91 - Sable Island, Nova Scotia
	stranding	1	? - Gros Morin Nat'l Park, Newfoundland
	stranding	2	<?/?/76 - the wash, eastern England
	stranding	1	?/?/90 - Liverpool, England
	stranding	4	?/?/92 - western Scotland
TOTAL	25	59	

TABLE 2 : Short-finned pilot whale samples - All available information is included in the table. A "?" is present wherever information about the date of sample acquisition is not included in source institution records.

	EVENT	# WHALES	DATE & LOCATION
	stranding	1	4/7/78 - North Carolina, 3517N/7521W
	stranding	1	4/12/78 - North Carolina, 3547N/7532W
	stranding	1	5/18/83 - North Carolina, 3622N/7549W
	stranding	1	11/11/91 - Delaware, 3839N/7504W
	bycatch	2	11/8/90 - 3625N/7443W
	bycatch	2	11/8/90 - 3633N/7441W
	bycatch	1	11/17/90 - 3656N/7435W
	bycatch	2	10/18/92 - western North Atlantic
	bycatch	1	9/27/90 - ETP, 0720N/8205W
	bycatch	1	7/28/92 - southern CA, 3515N/12234W
TOTAL	8 Atlantic	11 Atlantic	
	2 Pacific	2 Pacific	

TABLE 3 : Bottlenose dolphin samples - All available information is included in the table. A "?" is present wherever information about date of sample acquisition is not included in source institution records.

	EVENT	# WHALES	DATE & LOCATION
	bycatch	1	4/20/89 - ?
	bycatch	1	?/?/89 - ?
	bycatch	1	10/3/89 - 4029N/6659W
	bycatch	1	10/3/89 - 4030N/6657W
	bycatch	1	10/7/89 - 4016N/6750W
	bycatch	1	8/24/91 - 4001N/7026W
	bycatch	1	4/24/92 - ?
	bycatch	1	1/5/93 - ?
	bycatch	1	?/?/93 - ?
	bycatch	2	10/4/89 - 4029N/6703W
TOTAL	10	11	

(1989) using published sequences from human, cow, and mouse mtDNA (Anderson et al. 1981, Anderson et al. 1982, Bibb et al. 1981). Four other primers were used to amplify portions of the D-loop. Two of these primers anneal to highly conserved regions within the proline and phenylalanine tRNAs which flank the D-loop (tRNApro and tRNAphe) and were designed using published human, cow, and mouse mtDNA sequences (Anderson et al. 1981, Anderson et al. 1982, Bibb et al. 1981). The last two primers were modified from primers described in Shields & Kocher (1991) using published sequences for the cyt *b* gene in *Stenella* spp. (Irwin et al. 1991) and the D-loop in the Commerson's dolphin (*Cephalorhynchus commersonii*) (Southern et al. 1988). These primers (cytbL15792 and DloopDH) were used to amplify the 5' end of the D-loop, the proline tRNA, and the 3' end of the cyt *b* gene. The 5' and 3' designations refer to the light strand of the mtDNA.

DNA amplifications were carried out in 50 *u*l or 100 *u*l volumes using reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, and 1% Triton X-100) and 1 to 1.25 units of *Taq* polymerase from Promega. Reactions also contained 200 *u*M dGTP, dATP, dCTP, and dTTP, 3 mM $MgCl_2$, and primer concentrations of 0.2 *u*M each in double-stranded reactions and 1 *u*M and 0.02 *u*M in single-stranded reactions. Double-stranded PCR products were isolated in a 3% Nu-sieve gel in TA buffer, and the appropriate band was cut out of the gel and remelted in 200-750 *u*l of sterile distilled water. In most cases, 1 *u*l of this bandmelt was used in a single-stranded PCR reaction using an unbalanced primer ratio, where the concentration of one primer in the reaction was fifty times as high as the concentration of the other primer. A 350 -bp single-stranded cyt *b* gene fragment was synthesized using the two

TABLE 4 : Primer sequences - The top sequences are the primers used in this study, and they are aligned with the published fin whale (*Balaenoptera physalus*), cow, human, and mouse sequences for the same regions (Anderson et al. 1981, Anderson et al. 1982, Árnason et al. 1991, Bibb et al. 1981). Dots represent nucleotides in the latter sequences that are identical to the corresponding nucleotides in the top sequence. The sequences shown are from the 5' to 3' end of the strand indicated. Redundancy codes used in mixed probes are: Y=T+C, R=G+A, M=A+C.

cytbL26 (light)	CCATCCAACATCTCAGCATGATGAAA	
	.....A..T.....TT.......G..	fin whale
	....A.....T...T...........	cow
	............C............	human
	..........T...T..........	mouse
cytbH24 (heavy)	CCCTCAGAATGATATTTGTCCTCA	
	G..........................	fin whale
	T..........................	cow
	....	human
	A..........................	mouse
cytbL15792 (light)	ATTGTAGGCCAATTAGCATC	
	............C.A.....	fin whale
	.CCA.C..A...C.....	cow
	..CA.T..A...G.....	human
	..CA.T......C....C..	mouse
DloopDH (heavy)	CCTGAAGTAAGAACCAGATG	
	....	fin whale
	.......A............	cow
	.........G..........	human
	....	mouse
tRNApro (light)	CACCAYYARCMCCCAAAGCT	
	.....TC.G.A.........	fin whale
	.....TC.A.C.........	cow
	.....TT.G.A.........	human
	.....CC.G.A.........	mouse

cyt *b* gene primers. The D-loop amplifications were carried out in one of two ways. Whenever possible, primers cytbL15792 and DloopDH were used synthesize a 750-bp double-stranded PCR product. The DloopDH primer and the tRNApro primer, which annealed to a site within the double-stranded PCR product, were used to synthesize a 500-bp single-stranded fragment. Whenever the isolated DNA was of low quality, the double-stranded and single-stranded amplifications were carried out using the DloopDH and tRNApro primers. The sequences from the single-stranded templates created using the 750-bp cytbL15792-DloopDH double-stranded products were often much clearer than those from the single-stranded templates created using only the DloopDH and tRNApro primers, but I was often unable to amplify the larger 750-bp fragment from the low quality DNA.

The single-stranded DNA synthesized using PCR was directly sequenced using a modified Sanger chain termination sequencing method (United States Biochemical *Sequenase®* protocol). The primers used in the sequencing reactions were those with the lower concentration during the single-stranded PCR amplifications. In order to obtain accurate sequences, both the light and the heavy strand were sequenced for each individual. Sequencing reactions were carried out using reagents from the *Sequenase®* kit and approximately 0.3-1 *p*mole of template and 3 *p*moles of primer. Annealing reactions, containing template, primer, and reaction buffer (40 mM Tris-HCl pH7.5, 20 mM MgCl2, and 50 mM NaCl) were heated in boiling water for 2 minutes and quickly cooled by placing the reactions on ice for at least 1 minute. Condensation was removed from the sides and top of the reaction tubes by centrifugation, and 6.6 *u*l of sequencing mix (20 mM DTT, 3.2 mM Tris-HCl pH 7.5,

0.16 mg/ml bovine serum albumin, 6 *u*Ci ^{35}S-dATP, and 7.8 units of Sequenase enzyme) was added to each annealing reaction. After mixing, 3.5 *u*l of the resulting sequencing reaction was immediately aliquoted to each of four tubes containing 2.5 *u*l of ddGTP, ddATP, ddTTP, or ddCTP termination mixes. These tubes were incubated at 37°C for 2-3 minutes before the reactions were completed by adding 4 *u*l of stop mix. If the reactions were not immediately loaded onto a gel, they were stored at -20°C. Reactions were heated for 2-5 minutes at 75-80°C before loading them onto a 6% polyacrylimide gel. Sequence ladders were visualized using autoradiography (Kodak XAR film).

Analysis of Mitochondrial DNA Sequence Data

The sequences on the exposed films were read by hand using a light box, and entered into a computer. I was able to determine 400-bp D-loop sequences and 303-bp cytochrome *b* gene sequences for my analyses. Alignments were done visually without the aid of any computer software, and transition:transversion ratios and pairwise sequence divergences (the percent difference in basepair composition between 2 sequences) were calculated. D-loop nucleotide diversity (π), a measure of the average number of nucleotide differences per site in a population, was also estimated to allow comparison to other studies using the formula

$$\pi = (n/n\text{-}1)\Sigma\, x_i x_j \pi_{ij}$$

where n = the number of individuals sampled from the population, x_i and x_j = the estimated population frequency of the i^{th} and j^{th} type of DNA sequence, and π_{ij} = the proportion of different nucleotides between the i^{th} and j^{th} type of DNA sequence (Nei

1987). To determine the frame of the pilot whale cyt *b* gene sequences, they were aligned with published cyt *b* gene sequences from spinner and bridled dolphins (*Stenella longirostris* and *S. attenuata*)(Irwin et al. 1991).

RESULTS

The D-loop sequences determined from all of the incidentally caught long-finned pilot whales, the long-finned pilot whales that stranded on Cape Cod, the long-finned pilot whales that stranded in Scotland, and the long-finned pilot whales that stranded in England were completely identical ("Cape Cod" whales). However, the D-loop sequences determined from the long-finned pilot whales that stranded in Newfoundland and on Sable Island, Nova Scotia showed some variability, differing from the Cape Cod long-finned pilot whale sequence by 1 transitional substitution and 2 transitional substitutions, respectively (Figure 2 and Table 5). All of the Atlantic short-finned pilot whales had identical D-loop sequences, and this sequence differed from the long-finned pilot whale sequences by 13-15 base substitutions (pairwise sequence divergence = 3.25-3.75%)(Figure 2 and Table 5). Transition:transversion ratios were all greater than 12:1 indicating a lack of multiple substitutions, so pairwise sequence divergences were calculated without any corrections. Finally, the two Pacific short-finned pilot whale sequences differed from each other by one transitional substitution, from the Atlantic short-finned pilot whale sequence by 1 or 2 transitional substitutions, and from the long-finned pilot whale sequences by 11-14 base substitutions (pairwise sequence divergence = 2.75-3.5%)

(Figure 2 and Table 5). Once again, since all transition:transversion ratios were greater than 10:1, no corrections were made for multiple hits. Nucleotide diversity in long-finned pilot whales was 0.03% and in short-finned pilot whales was 0.05%.

TABLE 5 : Number of basepair differences, shown as number of transitions:number of transversions (above diagonal), and pairwise sequence divergences (below diagonal) for 400-bp D-loop sequence comparisons between pilot whale populations and species. For details about pilot whale groups, see Figure 2.

Gmel-CES = long-finned pilot whales, *Globicephala melas*, from Cape Cod, England, and Scotland
Gmel-New = long-finned pilot whales, *G. melas*, from Newfoundland
Gmel-NS = long-finned pilot whales, *G. melas*, from Nova Scotia
Gmac-A = short-finned pilot whales, *G. macrorhynchus*, from Atlantic
Gmac-P1, Gmac-P2 = short-finned pilot whales, *G. macrorhynchus*, from Pacific

	Gmel-CES	Gmel-New	Gmel-NS	Gmac-A	Gmac-P1	Gmac-P2
Gmel-CES	***	1:0	2:0	12:1	11:1	10:1
Gmel-New	.0025	***	1:0	13:1	12:1	11:1
Gmel-NS	.005	.0025	***	14:1	13:1	12:1
Gmac-A	.0325	.035	.0375	***	1:0	2:0
Gmac-P1	.03	.0325	.035	.0025	***	1:0
Gmac-P2	.0275	.03	.0325	.005	.0025	***

The cytochrome *b* gene sequences I determined for 16 long-finned pilot whales from all sampled locations, 4 Atlantic short-finned pilot whales, and two Pacific short finned pilot whales were all identical within each group, and differed from each other by 1-3 transitional substitutions (% sequence divergence = 0.33-0.99%)(Figure 3 and Table 6). Surprisingly, all substitutions were in the first position of codons. Although two substitutions were synonymous, the third substitution resulted in an amino acid change from glycine to serine (R=H to R=OH).

TABLE 6 : Number of basepair differences, shown as number of transitions:number of transversions (above diagonal), and pairwise sequence divergences (below diagonal) for 303-bp cytochrome *b* gene sequence comparisons between pilot whale populations and species. For details about pilot whale groups, see Figure 3.

Gmel = long-finned pilot whales, *Globicephala melas*
Gmac-A = short-finned pilot whales, *G. macrorhynchus*, from Atlantic
Gmac-P = short-finned pilot whales, *G. macrorhynchus*, from Pacific

	Gmel	Gmac-A	Gmac-P
Gmel	***	3:0	2:0
Gmac-A	.0099	***	1:0
Gmac-P	.0066	.0033	***

D-loop sequences were also determined from 11 bottlenose dolphins from 10 incidental catches in the western mid- North Atlantic, and I found that all of the individuals had different D-loop sequences, with pairwise sequence differences of 1 to 17 base substitutions (pairwise sequence divergence = 0.25-4.25%, average pairwise sequence divergence = 2.50%)(Figure 4, Figure 5, and Table 7). The nucleotide diversity was 1.25%. Two dolphins were caught together, and the sequence divergence within this pair was 3.50%.

TABLE 7 : Number of basepair differences, shown as number of transitions:number of transversions + number of deletions (above diagonal), and pairwise sequence divergences (below diagonal) for 400-bp D-loop sequence comparisons between 11 western North Atlantic bottlenose dolphins. Dolphins J and K were caught together.

	A	B	C	D	E	F	G	H	I	J	K
A	***	6:0	7:1	7:0	12:2	4:2	12:1+1	10:1	10:2	13:1	9:0+1
B	.015	***	6:1	5:0	9:2	5:2	11:1+1	9:1	10:1	11:1	5:0+1
C	.02	.0175	***	7:1	8:1	10:1	12:0+1	10:0	10:1	9:2	9:0+1
D	.0175	.0125	.02	***	6:2	2:2	12:1+1	8:1	8:2	8:1	6:0+1
E	.035	.0275	.0225	.02	***	8:0	12:1+1	4:1	4:0	2:1	10:2+1
F	.015	.0175	.0275	.01	.02	***	12:1+1	10:1	10:0	10:1	6:2+1
G	.035	.0325	.0325	.035	.035	.035	***	12:0+1	12:1+1	14:2+1	14:1+2
H	.0275	.025	.025	.0225	.0125	.0325	.0275	***	1:0	4:2	12:1+1
I	.03	.0275	.0275	.025	.01	.035	.025	.0025	***	4:1	12:2+1
J	.035	.03	.0275	.0225	.0075	.0275	.0425	.015	.0125	***	12:1+1
K	.025	.015	.0275	.0175	.0325	.0225	.0425	.035	.0375	.035	***

DISCUSSION

These results suggest that long-finned pilot whales from the eastern and western North Atlantic are not genetically isolated from each other, and that there is only one population of long-finned pilot whales in the North Atlantic. This population may have one dominant and at least two less common mtDNA haplotypes. Since I observed only three different D-loop sequences in the long-finned pilot whales I surveyed (n = 59, number of pods and bycatches sampled = 25), mtDNA variability in North Atlantic long-finned pilot whales may be unusually low. This conclusion is supported by additional sequences determined from 10 female Faroese pilot whales (Dizon et al. 1993). Nine of the ten whales had sequences that were identical to the "Cape Cod" sequence, and the tenth whale had a sequence that was identical to the Newfoundland sequence.

The D-loop is a non-coding region involved in replication, and it is commonly thought to be the most variable region in the mitochondrial genome. It has been estimated that the rate of D-loop evolution in cetaceans is 0.5%/Myr for the entire D-loop, with the rate outside the central region being higher (0.7%/Myr) (Hoelzel et al. 1991). Estimates for rates of D-loop sequence divergence in other mammals are as high as 10-20%/Myr in shrews (Stewart & Baker 1994), 8-17%/Myr in humans (Cann et al. 1987, Vigilant et al. 1989, 1991) and greater than 3.5%/Myr in kangaroo rats(Thomas et al. 1990). In humans, the D-loop appears to evolve at 4-5 times the rate of the entire mitochondrial genome (Cann et al. 1987).

Higher levels of intraspecific D-loop sequence variation have been described in other cetacean species. As reported in this study, eleven unique D-loop sequences were determined from eleven bottlenose dolphins taken in one region of the North Atlantic, and a nucleotide diversity of 1.25% was calculated. Similar studies on harbour porpoises (*Phocoena phocoena*) and common dolphins (*Delphinus delphis*) found nucleotide diversities ranging from 0.4% for North Atlantic harbour porpoises to 0.78% for short-beaked common dolphins off the coast of California (Rosel 1992).

It has been suggested that populations which exhibit extremely low levels of mtDNA variability have gone through severe population bottlenecks in the recent past (Wilson et al. 1985). MtDNA appears to be maternally inherited, and individuals are usually homoplasmic (for exceptions, see chapter 1). The effective number of mitochondrial genes is 1/4 that of nuclear genes, making mtDNA very sensitive to decreases in population size. The loss of mtDNA variation in a cetacean species with a small effective population size has been reported for vaquitas (*Phocoena sinus*), when a 412-bp D-loop sequence determined from 15 vaquitas showed no variation (Rosel 1992). The range of the vaquita is limited to the upper Gulf of California, and current population estimates for this species are very low. She hypothesized that this low level of mitochondrial genetic variability was caused by random genetic drift in a species with a long-term small effective population size or by a population bottleneck during recent (15,000 years ago) Pleistocene glacial periods when the species was founded by isolated individuals from a closely related species. However, pilot whales are a pelagic species that can disperse long distances, and current population estimates suggest that there are more than 700,000 long-finned pilot whales in just the

eastern North Atlantic (Buckland et al. in press). Since they were not heavily hunted throughout their range, it is highly unlikely that the North Atlantic population passed through a severe population bottleneck. Consequently, in order to explain my results, I have hypothesized that the low levels of mtDNA variability observed in pilot whales may be a result of a slow rate of sequence evolution or metapopulation dynamics resulting from the social system of pilot whales.

Slow Rate of Mitochondrial DNA Sequence Evolution

Short-finned pilot whales are found in tropical and warm temperate waters, and the Atlantic and Pacific populations of this species are separated by the Panamanian land bridge. Since this land bridge closed approximately 3 million years ago (Keigwin 1982), Atlantic and Pacific short-finned pilot whales have been separated for at least this long. Using a published rate of D-loop sequence evolution in cetaceans of 0.5%/Myr (Hoelzel et al. 1991), this duration of separation would suggest a D-loop sequence divergence between Atlantic and Pacific short-finned pilot whales of approximately 1.5%. My sequence analyses showed that there is only a 0.25% sequence divergence between Atlantic and Pacific short-finned pilot whale D-loop sequences (after correction for within-group divergence). This is one sixth of the expected value calculated using a conservative rate of D-loop evolution which is lower than estimated rates in other mammals (Cann et al. 1987, Stewart & Baker 1994, Thomas et al. 1990, Vigilant et al. 1989, 1991).

Slow rates of mtDNA sequence evolution have been reported for turtles (Avise et al. 1992, Bowen et al. 1992) and sharks (Martin et al. 1992), and it has been

suggested that these slow rates might be correlated with the low metabolic rates of these species. However, marine mammals have metabolic rates that are comparable to terrestrial mammals, and these rates are at least 5-10 times those of amphibians or fish (Huntley 1987, Lavigne 1982). On the other hand, close similarity of Atlantic and Pacific mitochondrial genotypes have been observed in sea urchins (Palumbi & Kessing 1991, Palumbi & Wilson 1990) and tuna species (Graves & Dizon 1989, Graves et al. 1984). In these latter cases, genetic similarities between animals from the Atlantic and Pacific were attributed to gene flow between the oceans via a trans-Arctic or southern ocean exchange. There have been no reported sightings of short-finned pilot whales in Arctic waters or southern waters near the Cape of Good Hope or Cape Horn. However, the possibility of gene flow between Atlantic and Pacific short-finned pilot whales cannot be completely dismissed.

Metapopulation Dynamics

Since mtDNA is maternally inherited, the behavior of females determines mitochondrial gene flow. Pilot whale pods seem to consist of a stable core of related females and their offspring, and female pilot whales do not appear to disperse from their natal pods (Amos et al. 1991a, 1991b, 1993). Consequently, mitochondrial gene flow between pods does not occur because they are behaviorally subdivided. Because mtDNA types belonging to a single female lineage are identical unless a mutation has occurred, most pods include only one inheritable mtDNA type. Consequently, mitochondrial genetic variation is found primarily between pods. Pilot whales were and, in some places, still are frequently harvested as entire pods (Andersen 1988,

Mitchell 1975a, 1975b, Sergeant 1962), and because of their socially cohesive behavior, they mass strand (Sergeant 1982) and may experience disease epidemics that affect entire pods (Harwood & Hall 1990). These pod extinctions could result in loss of rare mtDNA haplotypes.

If pod extinctions were frequent, they could significantly decrease mitochondrial genetic variability through a mechanism other than loss of rare mtDNA types. Computer simulation and theoretical studies have shown that lower than expected average heterozygosity and even apparent bottleneck effects might be seen in subdivided populations that never experienced significant overall decreases in population size (Gilpin 1991, Maruyama & Kimura 1980). Genetic variability can be lost in a metapopulation, or population of sub-populations, when sub-populations frequently become extinct and the areas they inhabited are recolonized by other sub-populations within the metapopulation. Although these studies focused on populations that are geographically subdivided, a pilot whale population can be described as a metapopulation of many sympatric but behaviorally subdivided sub-populations of females. Frequent extinction of pods, followed by growth and subdivision of surviving pods, could result in the loss of mtDNA variability in a pilot whale population.

This hypothesis is supported by a comparison of mitochondrial genetic variation found in Delphinid species with social systems that are similar to or different from that of pilot whales. Killer whales, like pilot whales, have a social system characterized by stable family groups where offspring stay with their mothers (Bigg 1982, Heimlich-Boran 1986). Although complete D-loop sequences have been

used to distinguish between sympatric, but behaviorally isolated, killer whale communities off Vancouver Island, overall levels of D-loop variability appear to be very low (Hoelzel 1991, Hoelzel & Dover 1991). In this study, a 362-bp sequence from the 5'-end of the D-loop, contained within the region I sequenced was analyzed for killer whales from widely separated geographical locations. The sequences differed by only 2 basepairs (pairwise sequence divergence = 0.55%) between whales from Vancouver Island and Iceland and were identical between whales from Vancouver Island and the North Sea off Germany. Nucleotide diversity among 9 killer whales within this region of the D-loop was 0.25%, while nucleotide diversity in pilot whales from this study was 0.03-0.05%.

In contrast, some Delphinid species have social systems characterized by fluid social groups and offspring that do not remain with their mothers after reaching maturity. Examples include bottlenose dolphins, common dolphins, and spinner dolphins. Studies on these species reveal much higher levels of mitochondrial genetic variability. In addition to my results from bottlenose dolphins, which showed significant D-loop sequence variation between dolphins from one geographical area, D-loop sequence variation has been examined in common dolphins (Rosel 1992), and whole mitochondrial variation has been surveyed using restriction enzymes in spinner dolphins (*Stenella longirostris*)(Dizon et al. 1991) and bottlenose dolphins (Dowling & Brown 1993). In all of these studies, multiple mtDNA sequences were found within populations, and levels of mitochondrial variation were much higher than those found in studies of pilot whales or killer whales. For instance, nucleotide diversity among 11

bottlenose dolphins from this study was 1.25%, and nucleotide diversity among 29 common dolphins within the same region of the D-loop was 2.10% (Rosel 1992).

I have developed a computer model to examine the effect of social structure on mtDNA variation in pilot whales more rigorously. In the model simulations, mtDNA diversity is monitored in a population of pilot whales under different scenarios, where extinction and mutation rates and pod dynamics can be altered. Details on the computer program and simulation results can be found in Chapter 3.

Mitochondrial DNA Variation as a Tool for Examining Social Structure

Genetic variation has been found in long-finned pilot whale populations using methods that examine nuclear DNA, such as protein electrophoresis (Andersen 1988), DNA fingerprinting (Amos et al. 1991a, 1991b), and microsatellite analysis (Amos et al. 1993). Therefore, the results of my mtDNA sequencing cannot be interpreted as evidence that North Atlantic long-finned pilot whales lack genetic variation. Instead, the results of this study demonstrate that although mtDNA is not a useful tool for examining the genetic stock structure of pilot whales, it does provide supporting evidence for the social structure of this species.

Early studies on mtDNA noted that patterns of variation would reflect the behavior of females (Lansman et al. 1981). Consequently, when the dispersal patterns of males and females are not identical, patterns of nuclear and mitochondrial genetic variation may differ as well. In species where males disperse farther than females, patterns of mtDNA variation might be found even if surveys using nuclear DNA markers show little or no genetic differentiation. For instance, tagging studies have

shown that female green turtles return to their natal rookeries to nest, and if turtles are sampled at rookeries, females appear to not disperse. MtDNA analyses on green turtles revealed geographic patterns of genetic variation that correspond to rookeries (Bowen et al. 1992). However, examination of nuclear DNA markers showed that common nuclear haplotypes were found in most rookeries, suggesting that male-mediated gene flow does occur in this species (Karl et al. 1992).

This work indicates that in species where the behavior of females and males differ, not only the patterns but also the levels of mitochondrial versus nuclear variability might be affected. Clearly, the results of mtDNA surveys must be interpreted with caution. Yet, they might also provide a method for obtaining initial evidence concerning the social structure of other marine mammal species that cannot be easily studied in other ways, particularly if mitochondrial and nuclear genetic markers are utilized.

FIGURE 1 : Primer locations - This schematic shows the approximate locations of the five primers used for amplification and sequencing of pilot whale and bottlenose dolphin mitochondrial DNA. The arrows point in the direction of primer extension during PCR and sequencing reactions. The numbers indicate the following primers: 1-cytbL26, 2-cytbH24, 3-cytbL15792, 4-tRNApro, 5-DloopDH.

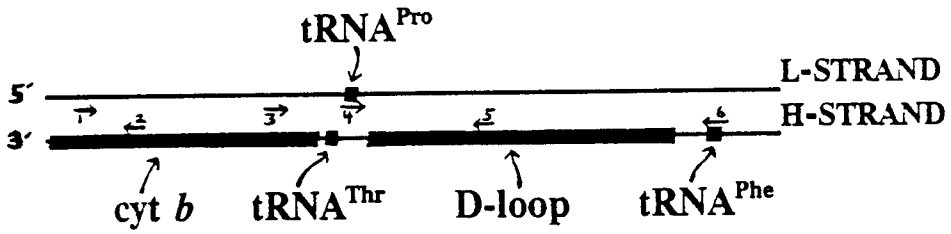

FIGURE 2 : Alignment of 400-bp pilot whale D-loop sequences. The sequences shown are from the 5' to 3' end of the light strand, starting just upstream of $tRNA^{Pro}$. Dots represent nucleotides in the latter sequences that are identical to the corresponding nucleotide in the top sequence.

Gmel-C : *Globicephala melas* (Cape Cod) - 48 pilot whales from 4 strandings on Cape Cod and 16 bycatches in the western mid- North Atlantic (1989-1993)

Gmel-E : *Globicephala melas* (England) - 3 pilot whales from a stranding in western England (Liverpool) (1990) and a stranding in eastern England (the wash)(<1976)

Gmel-S : *Globicephala melas* (Scotland) - 4 pilot whales from a stranding in western Scotland (1992)

Gmel-New : *Globicephala melas* (Newfoundland) - 1 pilot whale from a stranding in western Newfoundland (Gros Morin) (1990s)

Gmel-NS : *Globicephala melas* (Nova Scotia) - 3 pilot whales from a stranding on Sable Island (1990s)

Gmac-A : *Globicephala macrorhynchus* (Atlantic) - 11 pilot whales from 4 individual strandings in Delaware or North Carolina (1978, 1983,1991) and 4 bycatches in the western mid- North Atlantic (1990,1993)

Gmac-P1 : *Globicephala macrorhynchus* (Pacific 1) - 1 pilot whale from a bycatch in the ETP (1990)

Gmac-P2 : *Globicephala macrorhynchus* (Pacific 2) - 1 pilot whale from a bycatch off California (1992)

```
AAAAGCTTAT TGTATAATTA CCACAACATT ACAGTACTAT GTCAGTATTA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

AAAGTAATTT ATTTTAAAAA CATTTTACTG TACACATCAC ATACGTATAT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ...TA.....
.......... .......... .......... .......... ...TA.....
.......... .......... .......... .......... ...TA.....

ACACGTACAC ATACCAATAT CTAGTCTTTC CTTATAAATA TATATATATA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.T........ .......... .......... .......... .C........
.T........ .......... .......... .......... .C........
.T........ .......... .......... .......... .C........

CATGCTATGT ATTACTGTGC ATTCATTTAT TTTCCATACG ATAAGTTAAA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... G.........
.......... .......... .......... .......... G.........
.......... .......... .......... .......... G.........

GTCCGTATTA ATTATCATTA ATTTTACATA TTACATAATA TGTATACTCT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.C........ .......... .......... .......... .....G....
.C........ .......... .......... .......... .....G....
.C........ .......... .......... .......... .....G....

TACATATTAT ATATCCCCTA ACAATTTTAT TTCCATTATA TCCTATGGTC
.......... .......... .T........ .......... ..........
.......... .......... .T.G...... .......... ..........
.......... ......T... .........C C......... ..........
.......... ......T... .........C C......... ..........
.......... ......T... .........C .......... ..........

GCTTATATTA GACCACGAGC TTTATCACCA TGCCGCGTGA AACCAGCAAC
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

CCGCTCGGCA GGGATCCCCC TTCTCGCACC GGGCCCATAC ATCGTGGGGG  Gmel-C,E,S
.......... .......... .......... .......... ..........  Gmel-New
.......... .......... .......... .......... ..........  Gmel-NS
.......... .........T. .......... .........T .C........  Gmac-A
.......... .........T. .......... .......... .C........  Gmac-P1
.......... .........T. .......... .......... .C........  Gmac-P2
```

FIGURE 3 : Alignment of 303-bp pilot whale cytochrome *b* gene sequences. The sequences shown are from the 5' to 3' end of the light strand, from position 14580 to position 15142 of the human sequence (Anderson et al. 1981). Dots represent nucleotides in the latter sequences that are identical to the corresponding nucleotide in the top sequence. The codon change from CTA to TTA results in no amino acid change, while the codon change from AGC to GGC results in an amino acid change from glycine (R=H) to serine (R=OH).

Gmel-C : *Globicephala melas* (Cape Cod) - 6 pilot whales from 1 stranding on Cape Cod and 3 bycatches in the western mid-North Atlantic (1989-1991)

Gmel-E : *Globicephala melas* (England) - 2 pilot whales from a stranding in western England (Liverpool) (1990) and a stranding in eastern England (the wash)(<1976)

Gmel-S : *Globicephala melas* (Scotland) - 4 pilot whales from a stranding in western Scotland (1992)

Gmel-New : *Globicephala melas* (Newfoundland) - 1 pilot whale from a stranding in western Newfoundland (Gros Morin) (1990s)

Gmel-NS :*Globicephala melas* (Nova Scotia) - 3 pilot whales from a stranding on Sable Island (1990s)

Gmac-A : *Globicephala macrorhynchus* (Atlantic) - 4 pilot whales from 4 individual strandings in Delaware or North Carolina (1978, 1983, 1991)

Gmac-P : *Globicephala macrorhynchus* (Pacific) - 2 pilot whales from 1 bycatch in the ETP (1990) and 1 bycatch off California (1992)

```
AAC TTT GGC TCC TTA CTA GGC CTC TGC CTA ATT ATA CAA ATC
... ... ... ... ... ... ... ... ... T.. ... ... ... ...
... ... ... ... ... ... ... ... ... T.. ... ... ... ...

CTA ACA GGT TTA TTC CTA GCA ATA CAT TAC ACA CCA GAC ACC
... ... ... ... ... ... ... ... ... ... ... ... ... ...
... ... ... ... ... ... ... ... ... ... ... ... ... ...

TCA ACC GCT TTT TCA TCA GTC GCA CAC ATC TGT CGA GAC GTC
... ... ... ... ... ... ... ... ... ... ... ... ... ...
... ... ... ... ... ... ... ... ... ... ... ... ... ...

AAC TAT GGC TGA TTC ATC CGC TAC CTA CAT GCA AAC GGA GCT
... ... ... ... ... ... ... ... ... ... ... ... ... ...
... ... ... ... ... ... ... ... ... ... ... ... ... ...

TCC ATA TTC TTC ATC TGC CTT TAC GCC CAC ATC GGA CGT AGC
... ... ... ... ... ... ... ... ... ... ... ... ... G..
... ... ... ... ... ... ... ... ... ... ... ... ... G..

TTA TAC TAT GGC TCT TAT ATA TTC CAA GAA ACA TGA AAC ATT
C.. ... ... ... ... ... ... ... ... ... ... ... ... ...
... ... ... ... ... ... ... ... ... ... ... ... ... ...

GGT GTG CTC CTA CTA CTA ACA GTC ATG GCC ACT GCA TTC GTA
... ... ... ... ... ... ... ... ... ... ... ... ... ...
... ... ... ... ... ... ... ... ... ... ... ... ... ...

GGC TAT GTC   Gmel-C,E,S,New,NS
... ... ...   Gmac-A
... ... ...   Gmac-P
```

FIGURE 4 : 400-bp D-loop sequence from 11 western North Atlantic bottlenose dolphins. The sequence shown is from the 5' to 3' end of the light strand, starting just upstream of $tRNA^{Pro}$. The variable sites in the sequence are represented by lower case letters as follows: n=G,A,T,C; y=T,C; r=G,A; m=A,C; a=A, deletion; c=C,deletion.

```
AAAGCTTATT GTACAGTTAC CACAACAyCA CAGTACTAyn TCAGTATTAA

AAGTAATTTG TTTTAAAAAC ATTTTACTGT ACACATTACA TAyACATACr

CATGyrCATG CTAATATTTA GTCTCTCCTT GTAAATATTC ATAyATACAT

GCTATGTATT ATTGTGCATT CATTTATTTT CCATACGAyA AGTTAAAGCy

CGTATTAATT ATCATTAATT TTACATATyA CATAATATGy ATGCTCTTAC

ATATTATATm TCCYCTaTcA ATyyTAyyyC CATTATAyyC TATGGTCrCT

CCATTAGATC rCGAGCTTAA TCACCATGCC GCGTGAAACC AGCAACCCGC

TyGGCAGGGA TCCCTCTTCT CGCACCGGGC CCATAyCTCG TGGGGGTAGC
```

FIGURE 5 : The 27 variable sites in a 400-bp D-loop sequence from 11 western North Atlantic bottlenose dolphins. The bottom two sequences are from two dolphins that were caught together.

```
T C C C A T G C C C T T A T A C C T T C T T T G A T C
. . T T . . . . T T . . . C . . . . C . . . . . . . .
. T A T . . . . T . . C . C . . . C . . . . . . . C .
C . T T . . . . . . . . . C . . . . C . . . C . . C .
C . G T . C . T T . . C C C . . . C C . . . C A . C .
C . G . . . . . . . . . C C . . . . C . . . C . . . .
. . G . . . A T T T . C . C - . T . . T C C C . . C .
C . G T . C . T T T . . . . . . . C . . . . C A . C .
C . G T . C . T T T . . C . . . . C . . . . C A . C .
C . T T . C . T T . . C C . . . . C C . . . C A . C T
. . T T G . . . T . C . . C . - . . C . . . C . G . .
```

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CHAPTER THREE

An Individual-based Model of Mitochondrial Genetic Diversity in a Behaviorally Subdivided Marine Mammal Population and Preliminary Results on the Effect of Pod Extinctions

INTRODUCTION

The North Atlantic long-finned pilot whale, *Globicephala melas*, is a pelagic cetacean species with a highly structured social system, and genetic studies have found that mitochondrial DNA (mtDNA) sequence variability in North Atlantic pilot whales may be unusually low (chapter 2, Dizon et al. 1993). The ranges of pelagic cetaceans, such as the pilot whale, are not limited by geographic barriers within an ocean basin. However, mitochondrial gene flow within a pilot whale population is limited due to behavioral constraints. Since mtDNA is maternally inherited, the behavior of females determines mitochondrial gene flow. Pilot whale pods seem to consist of a stable core of related females and their offspring, and female pilot whales do not appear to disperse from their natal pods (Amos et al. 1991a, 1991b, 1993). Consequently, mitochondrial gene flow between pods does not occur because they are behaviorally subdivided.

Patterns of genetic variation in a population can be significantly affected by subdivision of the population, particularly if the resulting sub-populations are ephemeral (Gilpin 1991, Lande 1992, Maruyama & Kimura 1980, McCauley 1991,

Slatkin 1977, Wade & McCauley 1988). Although past studies have focused on populations which are geographically subdivided, similar effects could be seen in sympatric populations which are effectively subdivided because of behavioral constraints. The model described in this paper was designed to study the effect of pod extinction on mitochondrial genetic variation in a pilot whale population which is subdivided as a result of this species' social system.

Neutral gene theory predicts that in a finite population where mutations always result in new alleles, the frequencies of selectively neutral alleles are determined by a balance between accumulation of alleles through mutation and loss of alleles through random genetic drift (Kimura & Crow 1964). This theoretical model of mutation, known as the infinite allele model, can overestimate the expected heterozygosity in a subdivided population. Maruyama and Kimura (1980) considered the effect of random extinction and subsequent replacement of haploid subpopulations on heterozygosity in a metapopulation, or population of subpopulations. They showed that when subpopulation extinctions and replacements are frequent, the effective size of the metapopulation is reduced, causing the average heterozygosity of the metapopulation to be lower than expected using traditional neutral gene theory.

It has also been suggested that apparent bottleneck effects might be seen in subdivided populations which never experienced significant overall decreases in population size (Gilpin 1991, Pimm et al. 1989). Gilpin (1991) modeled the loss of heterozygosity in a metapopulation consisting of two or three subpopulations using a Monte Carlo simulation. He found that even if the size of the metapopulation

remained constant, heterozygosity in the metapopulation could eventually become zero after each subpopulation had undergone extinction and been replaced.

Pilot whales were and, in some places, still are frequently harvested as entire pods (Andersen 1988, Mitchell 1975a, 1975b, Sergeant 1962), and because of their socially cohesive behavior, they mass strand (Fehring & Wells 1976, Sergeant 1982) and may experience disease epidemics that affect entire pods (Harwood & Hall 1990). In addition, because pods do not grow to be infinitely large, it can be logically assumed that large pods split and could thereby replace pods lost to fisheries, stranding, or disease. However, because individuals in a pilot whale population are grouped into pods and because their behavior can be influenced by the behavior of other whales, they are not independent. Consequently, models such as those of Maruyama and Kimura (1980) and Gilpin (1991), which do not consider interactions between individuals, could not be applied to study the effect of pod extinction, growth, and splitting on the dynamics of mtDNA diversity in a population of pilot whales. It was necessary to design an individual-based model that tracked each pilot whale on the basis of her mitochondrial haplotype and pod affiliation.

MODEL DESCRIPTION

The model was designed to examine changes in mtDNA sequence variation in a population of pilot whales. It assumes that mtDNA is maternally inherited and that individuals are homoplasmic. Because only females transmit their mtDNA to their offspring, all whales in the modelled populations were females. Genetic variation was

measured as heterozygosity (h), which can be defined as the probability that the mitochondrial haplotypes of two randomly chosen individuals are different. It is calculated as $1 - \Sigma p_i^2$, where p_i is the population frequency of the ith mitochondrial haplotype. Mitochondrial haplotypes were modeled as 32 bit binary strings, because these were the longest strings which could be easily manipulated by the computer. All of the simulations ran for one million years.

Starting Conditions

The simulations were initiated with one of two possible starting conditions: a low heterozygosity condition with h set to zero and a high heterozygosity condition with h set to 0.95. The low heterozygosity cases began with a pod of 20 whales, all having the same mitochondrial haplotype with the 32 bits set to zero. The high heterozygosity cases started with a pod containing 20 haplotypes divided evenly among 100 whales. The mitochondrial haplotypes at the start of these simulations were created by setting one of the first 20 bits in the 32 bit string to one.

Population Growth

Density dependent population growth was incorporated into the model by varying the probability that an individual whale would give birth. In all of the simulations, the probability that an individual would die during each year was set to 0.05. The probability that an individual would give birth during that year, b, was defined as $0.05[(K-N)/K]+0.05$, where K was the carrying capacity of 100,000 whales and N was the population size, resulting in a logistic population growth curve (Figure

1). The death rate and initial birth rate were chosen because data on long-finned pilot whales from Newfoundland gives an annual mortality rate estimate of 3.5-5.8% and an annual birth rate estimate of 10-13% (Sergeant 1962). Birth and death events were determined by comparing the death and birth probabilities to a random number between zero and one. Death events occurred when the random number was greater than (1 - the death probability), and if the individual survived, birth events occurred when the random number was less than the birth probability. Using the same random number saved computational time, but caused the actual birth rate during simulations to be (b / 0.95).

Mutation

New mitochondrial haplotypes were created throughout the simulation by randomly changing one of the 32 bits. A mutation would occur during a birth event, if mutation probability, m, was greater than the random number used to determine birth and death events. Once again, this saved computational time, but resulted in a mutation rate of $u=m/b$ mutations/individual/year that was a function of the population size (Figures 1& 2). However, once the population reached demographic equilibrium after a few hundred years, u remained relatively constant. Therefore, values reported for u will refer to this equilibrium value.

Pod Splitting

Pods did not grow to be infinitely large. Pods would split into two according to a sigmoid probability curve defined by the equation $[(x/100)^8-(x/100)^4]/[(x/100)^8- 1]$,

where x was the pod size (Figure 3). Pods with 100 whales would split half of the time.

During the splitting process, haplotypes were divided between the two resulting pods using a splitting rule which subdivided the pods into monotypic groups of whales based on a splitting probability parameter, *c*. The whales of each haplotype in a pod were divided into groups of seven whales, with any remaining whales of that haplotype placed in a final smaller group of whales. Within each group of whales, one individual was randomly chosen as a "leader", and this whale had a 0.5 probability of staying in the pod and a 0.5 probability of leaving it based on a random number draw. All other whales in the group had a $(1+c)/2$ probability of following the leader.

The groups of seven whales sorted together during the splitting process were chosen to represent family groups. Data from killer whales (*Orcinus orca*) in Puget Sound has shown that pods are made up of subgroups of individuals with close associations and that these subgroups are matriarchal family groups (Heimlich-Boran 1986). Just considering females, a killer whale family group would typically consist of an older female, her daughters, and her granddaughters. Individual females and their offspring have been identified in well-studied killer whale pods and this information indicates that reproductive females have from 1 to 3 immature offspring (Balcomb & Bigg 1986). Thus, if a family group could be defined as an older female, her 1 to 3 daughters, and her daughters' 1 to 3 daughters, the average family group size would be seven whales.

Extinction

Pod-based or individual extinctions could occur during simulations after populations had expanded during the first 100 years. During pod-based extinction simulations, entire pods were removed at random at a rate of x pods/year. Individual extinction simulations were designed to randomly remove the same number of individuals that would have been removed at the same pod-based extinction rate. Because pods were different sizes, the individual extinction simulations operated by choosing a pod, as in the pod-based extinction simulations, counting the number of individuals in this pod, and then removing that number of individuals at random from the entire population.

Parameter Values

The following parameters were varied: the mutation rate u, the extinction rate x, and the splitting probability parameter c. All of the sets of parameter values were used with low and high heterozygosity starting conditions, and the effect of individual extinctions was tested for one set of parameter values.

The standard values were $u=1.73\text{x}10^{-5}$ mutations/individual/year, $x=1/100$ pod removed/pods/year, and $c=0.70$, and these parameter values were used in five simulations for each of the two starting heterozygosities. The same parameter values were also used in three simulations for each starting heterozygosity with individual extinction rather than pod-based extinction. In an additional three simulations for each starting heterozygosity, the pod extinction rate was set to zero. In other simulations, each one of the three parameters was varied independently to examine

the effect of the mutation rate, extinction rate, and splitting probability parameter. In one simulation for each starting heterozygosity, u was increased or decreased by a factor of ten, x was set to 1/10 or 1/1000 pod removed/pods/year, or c was set to zero. For a summary of this information, see Table 1.

The standard mutation rate, u, was chosen as a reasonable estimate for the mutation rate per individual per year for a 16,000-bp mtDNA where roughly 9600 basepairs are free to vary (Palumbi 1989). In mtDNA, it has been estimated that the mutation rate is $5x10^{-9}$ to $1x10^{-8}$ substitutions/site/year (Hartl & Clark 1989). Therefore the mutation rate/individual/year would be $4.8x10^{-5}$ to $9.6x10^{-5}$. The standard mutation rate of $u=1.73x10^{-5}$ mutations/individual/year was of the same order of magnitude.

The standard extinction rate of 1/100 pod removed/pods/year was chosen because it was within the range of fishery mortality rates reported for drive fisheries in Newfoundland and the Faroe Islands. Between 1951 and 1961, an average of 4280 whales per year were taken from an estimated population of 50,000-60,000 whales off Newfoundland (Mercer 1975, Mitchell 1975a). This was a loss of 1/12 whale taken/whales/year. The Faroese fishery has taken an average annual catch of approximately 400 to 1800 whales since records were kept starting in the late 1500s, and this population is now estimated to be approximately 778,000 whales (Buckland et al. in press, Mitchell 1975). This is a loss of approximately 1/2000 to 1/400 whale taken/whales/year. Because these two fisheries removed entire pods of whales rather than single whales, these fishery mortality estimates ranged from roughly 1/12 to 1/2000 pod removed/pods/year.

TABLE 1 : Parameter values used in simulations - N is the number of simulations completed with the listed parameters for each starting heterozygosity, u is the mutation rate/individual/year at demographic equilibrium, x is the fraction of pods removed/year, "type" is the method of extinction, and c is the splitting probability parameter. The first parameter values are the standard values, and the parameter value that is changed in the subsequent simulations is highlighted in boldface. No mutation rate is listed for the simulations with x=1/10 pods removed/year because these simulations crashed during the first 1000 years.

N	u	x	type	c
5	1.73×10^{-5}	1/100	pod	0.70
3	1.73×10^{-5}	1/100	**indiv.**	0.70
3	2×10^{-5}	**0**	pod	0.70
1	**1.73×10^{-4}**	1/100	pod	0.70
1	**1.73×10^{-6}**	1/100	pod	0.70
1	----------	**1/10**	pod	0.70
1	1.97×10^{-5}	**1/1000**	pod	0.70
1	1.73×10^{-5}	1/100	pod	**0**

The standard splitting probability parameter, *c*, was selected because of the assumption that pilot whales would remain with their closest relatives during a split. In a pod with more than one mitochondrial haplotype, close relatives would tend to have the same haplotype, and therefore, identical haplotypes would remain together during a split. If *c* was set to 1.0, whales in monotypic family groups would always stay together during a split because all whales in the group had a 100% chance of following the leader. With the standard value of $c = 0.70$, whales in a family group had an 85% chance of following the leader, accounting for the possibility that family groups might not always remain together. It should be noted that when *c* was reduced to zero, all whales in a pod had a 50% chance of staying in the pod and a 50% chance of leaving it, and pods were randomly split into two.

ANALYSIS OF GENETIC DIVERSITY

Descriptive Statistics

The mean heterozygosity was determined for each set of parameter values. To determine if different parameter values resulted in simulated populations that could have the low levels of mitochondrial genetic diversity observed in the North Atlantic pilot whale population, the percentage of time that simulated populations spent with heterozygosity below 0.14 was calculated. This value was used because three mitochondrial haplotypes have been found in the North Atlantic population, resulting in a heterozygosity of $1-[(64/69)^2+(3/69)^2+(2/69)^2]=0.137$ (chapter 2 and Dizon et al. 1993). The number of haplotypes was also monitored to assess the affect

of haplotype number versus relative haplotype frequencies on heterozygosity. To lessen the effects of starting conditions, descriptive summary statistics of heterozygosity and haplotype number were calculated for the last 500,000 years of the simulations.

Comparison to Infinite Allele Model

Because the modeled pilot whale populations were finite in size and there were 2^{32} (4,294,967,296) possible selectively neutral haplotypes, the assumptions of the infinite allele model of mutation were met. To determine if the results of the simulations agreed with the predicted results from population genetic theory, theoretical curves were developed using the recursion equation

$$F_t=(1-u)^2\{(1/2N_e)+[1-(1/2N_e)]F_{t-1}\}$$

where F_t is the probability of homozygosity at generation t, u is the average rate of mutation of alleles in a diploid population, and N_e is the effective population size (Kimura & Crow 1964). A generation is defined as the time it takes to introduce $2N_e u$ new mutants into the population. At equilibrium when $F_t=F_{t-1}$, $F=1/(4N_e u+1)$.

To use this equation to examine mtDNA variation, the number of females (N_f) can be substituted for $2N_e$ (Birky et al. 1983). Since all of the individuals in the simulated populations were females, the mean number of females was equivalent to the mean population size. Because homozygosity is defined as the probability that two randomly chosen alleles are identical, $1-F_t$ is the heterozygosity. Consequently, curves of $1-F_t$ versus time were used to compare the theoretical results from neutral gene theory to the simulation results.

RESULTS

Output from the model was reported every thousand years, and it included population size, the number of pods, pod sizes, the number of mtDNA haplotypes in the population, the number of pods with one or more than one haplotype, and the heterozygosity. Other output, such as the number of mutations per 1000 yrs and the number of extinctions per 1000 years, was used to confirm that the simulations were using the correct input parameters. Because setting the extinction rate to 1/10 pods removed/year caused loss of the population before year 999, no results will be reported for simulations with this parameter value. Throughout the results section of this paper, the units for the extinction rate (pod removed/pods/year) and the mutation rate (mutations/individual/year) will not be listed.

Genetic Diversity Summary Statistics

Examples showing the changes in mtDNA heterozygosity over time for simulations with different parameter values can be seen in Figures 4, 5, and 7 through 12.

Mean heterozygosity

The mean heterozygosities from simulations with different parameter combinations were compared using the Tukey multiple comparisons test for unequal sample sizes ($\alpha = 0.05$) available in SPSS (Norusis 1991). The results of this analysis showed that the mean heterozygosity in simulated populations during the last 500,000

years varied with changes in the starting heterozygosity, the extinction rate, the mutation rate, and the splitting probability parameter (Table 2).

When the starting heterozygosity was low, the mean heterozygosity under standard conditions (u=1.73x10^{-5}, x=1/100, and c=0.7) was 0.245, and during these simulations, the heterozygosity underwent large fluctuations, resulting in a standard deviation of 0.185. Applying the extinction rate of 1/100 to individuals rather than pods or reducing the pod extinction rate to 1/1000 increased the mean heterozygosity to 0.478 and 0.395, but decreased the magnitude of the fluctuations in heterozygosity, thereby decreasing the standard deviation to 0.135 and 0.087, respectively . When there was no extinction, the mean heterozygosity increased to 0.663 ± 0.117 (mean ± SD). Increasing the mutation rate to 1.73x10^{-4} increased the mean heterozygosity to 0.819 ± 0.058, and decreasing the mutation rate to 1.73x10^{-6} reduced the mean heterozygosity to 0.003 ± 0.013. When the splitting probability parameter was decreased from 0.7 to zero, the heterozygosity was near zero more often than it was under standard conditions, and the mean heterozygosity was reduced to 0.053 ± 0.117.

When the starting heterozygosity was high, the mean heterozygosity was 0.341 ± 0.218 under standard conditions. As when the starting heterozygosity was low, applying the extinction rate of 1/100 to individuals rather than pods increased the mean heterozygosity to 0.645 but decreased the standard deviation to 0.126 because large fluctuations in the heterozygosity did not occur. Likewise, if the pod extinction rate was decreased to 1/1000, fluctuations were small and while the mean heterozygosity increased to 0.621, the standard deviation decreased to 0.088. When

TABLE 2 : Summary statistics describing heterozygosity in simulations with different parameter values during the last 500,000 years of the simulations. u is the mutation rate/individual/year, x is the fraction of pods removed/year, c is the splitting probability parameter, N is the number of simulations completed with the listed parameters for each starting heterozygosity, "mean" is the mean heterozygosity for all simulations with the listed parameters (± the standard error of simulation means when applicable), and "SD" is the standard deviation of the heterozygosity about the mean for values from all simulations with the listed parameters. The first parameter values are the standard values, and in subsequent lines, the parameter values that differ from the standard value are highlighted in boldface. All groups in the table have significantly different heterozygosities (Tukey test, $\alpha = 0.05$).

Low starting heterozygosity

u, x, c	*N*	mean (±SE)	SD (range)
1.73×10^{-5}, 1/100, 0.7	5	0.245± 0.024	0.185 (2×10^{-5} - 0.775)
1.73×10^{-5}, **1/100(indiv)**, 0.7	3	0.478± 0.090	0.135 (0.073 - 0.730)
2×10^{-5}, **0**, 0.7	3	0.663± 0.109	0.117 (0.444 - 0.875)
1.97×10^{-5}, **1/1000**, 0.7	1	0.395	0.087 (0.165 - 0.573)
1.73×10^{-4}, 1/100, 0.7	1	0.819	0.058 (0.557 - 0.915)
1.73×10^{-6}, 1/100, 0.7	1	0.003	0.013 (0 - 0.120)
1.73×10^{-5}, 1/100, **0**	1	0.053	0.117 (0 - 0.513)

High starting heterozygosity

u, x, c	*N*	mean (±SE)	SD (range)
1.73×10^{-5}, 1/100, 0.7	5	0.341± 0.090	0.218 (0 - 0.725)
1.73×10^{-5}, **1/100(indiv)**, 0.7	3	0.645± 0.027	0.126 (0.233 - 0.834)
2×10^{-5}, **0**, 0.7	3	0.801± 0.044	0.059 (0.603 - 0.916)
1.97×10^{-5}, **1/1000**, 0.7	1	0.621	0.088 (0.432 - 0.786)
1.73×10^{-4}, 1/100, 0.7	1	0.767	0.086 (0.481 - 0.924)
1.73×10^{-6}, 1/100, 0.7	1	0.001	0.003 (0 - 0.029)
1.73×10^{-5}, 1/100, **0**	1	0.237	0.178 (2×10^{-5} - 0.655)

there was no extinction, the mean heterozygosity was 0.801 ± 0.059. Increasing the mutation rate to 1.73×10^{-4} increased the mean heterozygosity to 0.767 ± 0.086. When the mutation rate was decreased to 1.73×10^{-6}, the mean heterozygosity was reduced to 0.001 ± 0.003 and the heterozygosity frequently dropped to zero. As under low heterozygosity starting conditions, changing the splitting probability parameter to zero decreased the mean heterozygosity to 0.237 ± 0.178 because the heterozygosity was close to zero more often than under standard conditions.

Number of haplotypes

The mean numbers of haplotypes throughout the last 500,000 years of simulations were also analyzed using the Tukey multiple comparisons test for unequal sample sizes (α=0.05). This analysis showed that the number of haplotypes varied with changes in the starting heterozygosity, the extinction rate, the mutation rate, and when the starting heterozygosity was low, the splitting probability parameter (Table 3). Decreasing the pod extinction rate increased the number of haplotypes from roughly 6.5 when the extinction rate was 1/100 to greater than 20 when the extinction rate was zero. Applying the extinction rate of 1/100 to individuals also increased the number of haplotypes to greater than 10. Increasing the mutation rate to 1.73×10^{-4} increased the number of haplotypes to roughly 32, and decreasing the mutation rate to 1.73×10^{-6} decreased the number of haplotypes to approximately 1.5.

Although simulations with higher heterozygosities had higher numbers of haplotypes on average, the fluctuations in heterozygosity during simulations were not solely due to changes in the number of haplotypes. During simulations,

TABLE 3 : Number of haplotypes in simulations with different parameter values during the last 500,000 years of the simulations. x is the fraction of pod removed/pod/year, u is the mutation rate/individual/year, c is the splitting probability parameter, N is the number of simulations completed with the listed parameters for each starting heterozygosity, "mean" is the mean number of haplotypes for all simulations with the listed parameters (± the standard error of simulation means when applicable), and "SD" is the standard deviation of the haplotype number about the mean for values from all simulations with the listed parameters. The first parameter values are the standard values, and in subsequent lines, the parameter values that differ from the standard value are highlighted in boldface. All groups in the table have significantly different haplotype numbers (Tukey test, $\alpha = 0.05$).

Low starting heterozygosity

x	u	c	N	mean (±SE)	SD
1/100	1.73×10^{-5}	0.7	5	6.70± 0.35	2.01
1/100(indiv)	1.73×10^{-5}	0.7	3	10.32± 0.70	2.28
0	2×10^{-5}	0.7	3	23.20± 2.40	4.05
1/1000	1.97×10^{-5}	0.7	1	16.19	2.77
1/100	**1.73×10^{-4}**	0.7	1	32.91	3.92
1/100	**1.73×10^{-6}**	0.7	1	1.57	0.75
1/100	1.73×10^{-5}	**0**	1	5.57	1.80

High starting heterozygosity

x	u	N	mean (±SE)	SD
1/100	1.73×10^{-5}	6	6.54± 0.36	1.91
1/100(indiv)	1.73×10^{-5}	3	12.37± 0.41	2.64
0	2×10^{-5}	3	27.56± 2.59	4.64
1/1000	1.97×10^{-5}	1	18.51	3.06
1/100	**1.73×10^{-4}**	1	31.47	4.75
1/100	**1.73×10^{-6}**	1	1.55	0.73

heterozygosities spanning most of the range of observed values occurred throughout the range of observed haplotype numbers (Figure 6). Therefore, it appears that changes in heterozygosity were due primarily to variations in the relative frequencies of different haplotypes.

Time spent with heterozygosity below observed value

The amount of time simulated populations spent with heterozygosity below the observed value of 0.14 in the last 500,000 years was affected by the starting heterozygosity, the extinction rate, the mutation rate, and the splitting probability parameter (Table 4). Under low and high starting heterozygosity conditions, the heterozygosity never dropped below 0.14 during the last 500,000 years if the extinction rate was zero or 1/1000 or if the mutation rate was 1.73×10^{-4}. The heterozygosity during the last 500,000 years was always below 0.14 when the mutation rate was 1.73×10^{-6}. If the extinction rate of 1/100 was applied to individuals, the heterozygosity during the last 500,000 years was never lower than 0.14 when the starting heterozygosity was high and was lower than 0.14 less than 1% of the time when the starting heterozygosity was low. However, when the pod extinction rate was 1/100, the heterozygosity during the last 500,000 years was frequently below 0.14. When the splitting probability parameter was 0.7, this occurred 35% of the time when the starting heterozygosity was low and 27% of the time when the starting heterozygosity was high. Changing the splitting probability parameter to zero increased the percentage of time that the heterozygosity was below 0.14 during the last 500,000

TABLE 4 : Percentage of heterozygosity values below 0.14 during the last 500,000 years in simulations with different parameter values. *u* is the mutation rate/individual/year, *x* is the fraction of pods removed/year, *c* is the splitting probability parameter, *N* is the number of simulations completed with the listed parameters for each starting heterozygosity, and *h* is heterozygosity. The first parameter values are the standard values, and the parameter value that is changed in the subsequent simulations is highlighted in boldface. The observed heterozygosity in the North Atlantic pilot whale population was 0.137 (chapter 2, Dizon et al. 1993).

Low starting heterozygosity

u, x, c	*N*	*h* < 0.14
1.73×10^{-5}, 1/100, 0.7	5	35%
1.73×10^{-5}, **1/100(indiv)**, 0.7	3	0.9%
2×10^{-5}, **0**, 0.7	3	0
1.97×10^{-5}, **1/1000**, 0.7	1	0
1.73×10^{-4}, 1/100, 0.7	1	0
1.73×10^{-6}, 1/100, 0.7	1	100%
1.73×10^{-5}, 1/100, **0**	1	90%

High starting heterozygosity

u, x, c	*N*	*h* < 0.14
1.73×10^{-5}, 1/100, 0.7	5	27%
1.73×10^{-5}, **1/100(indiv)**, 0.7	3	0
2×10^{-5}, **0**, 0.7	3	0
1.97×10^{-5}, **1/1000**, 0.7	1	0
1.73×10^{-4}, 1/100, 0.7	1	0
1.73×10^{-6}, 1/100, 0.7	1	100%
1.73×10^{-5}, 1/100, **0**	1	34%

years to 90% when the starting heterozygosity was low and 34% when the starting heterozygosity was high.

Theoretical and Simulated Changes in Genetic Diversity

Two different theoretical curves were created for each starting heterozygosity: one curve for populations experiencing only demographic extinction (x=0) and one curve for populations experiencing pod or individual extinctions at the rate of 1/100 pod removed/pods/year. These curves were created using the mean population sizes and mutation rates at demographic equilibrium for simulated populations with these extinction rates. When the extinction rate was zero, the infinite alleles equation was solved with N_f=99,999 and $u=2\text{x}10^{-5}$. When the extinction rate was 1/100, the infinite alleles equation was solved with N_f=84,458 and $u=1.73\text{x}10^{-5}$. A generation in the infinite allele model was equivalent to the number of years it took to create $N\mu$ new mutant haplotypes. When the extinction rate was zero, a generation was 20 years, and when the extinction rate was 1/100, a generation was 16 years.

Looking at figures 7 through 12, it is clear that the general trend of changes in heterozygosity in simulated populations that experienced no extinction or individual extinctions was similar to the expected changes in heterozygosity predicted by the infinite allele model (Figures 7, 8, 10, and 11). However, heterozygosity in the simulated populations that experienced pod extinctions did not vary as predicted by the infinite allele model (Figures 9 and 12). Rather than gradually approaching the equilibrium heterozygosity, the genetic diversity in these populations underwent large fluctuations and frequently dropped to low values throughout the simulations.

TABLE 5 : Root mean square (RMS) error of the residuals between the observed heterozygosity in the simulated populations and the predicted heterozygosity using the infinite allele model for simulations with extinction rates (x) of zero, 1/100 pod removed/pods/year, or 1/100 pod removed/pods/year as individuals. The listed mutation rates (u) are the rates at the mean population size for those simulations, and these values were used to solve the infinite allele equation.

Low starting heterozygosity

x	u	RMS Error
0	$2x10^{-5}$	0.097
		0.125
		0.131
1/100 (indiv)	$1.73x10^{-5}$	0.151
		0.224
		0.263
1/100 (pod)	$1.73x10^{-5}$	0.316
		0.317
		0.366
		0.367
		0.391

High Starting heterozygosity

x	u	RMS Error
0	$2x10^{-5}$	0.042
		0.068
		0.074
1/100 (indiv)	$1.73x10^{-5}$	0.124
		0.148
		0.153
1/100 (pod)	$1.73x10^{-5}$	0.331
		0.381
		0.415
		0.468
		0.495

To test the goodness of fit between the predicted heterozygosity estimated using the infinite allele model (pred) and the observed heterozygosity in the simulated populations (obs), the root mean square (RMS) error was calculated for the residuals using the time series analysis package of SPSS (Norusis 1987). This statistic was defined as the square root of {[Σ (obs - pred)^2]/degrees of freedom}. Under low and high starting heterozygosity conditions, all of the RMS errors were highest for simulated populations that experienced pod extinctions and lowest for simulated populations that experienced no extinctions (Table 5).

Population Summary Statistics

Population size

The mean population sizes were compared using the Tukey multiple comparisons test for unequal sample sizes (α=0.05), and the variances about these

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TABLE 6 : Population sizes in simulations with different extinction rates. *x* is the extinction rate as the fraction of pods removed/year, *N* is the number of simulations with the listed extinction rate, "mean" is the mean population size for all simulations with the listed parameters (± the standard error of simulation means), and "SD" is the standard deviation of the population size about the mean for values from all simulations with the listed parameters. All groups in the table have significantly different population sizes (Tukey test, α = 0.05).

x	*N*	mean (±SE)	SD
0	6	99,998.9± 8.3	246.9
1/1000	2	98,474.9± 13.7	299.4
1/100(pod)	16	84,455.8± 14.8	666.1
1/100(indiv)	6	84,459.7± 13.7	568.3

mean sizes were compared using a test analogous to the Tukey test based on the natural logarithms of the variances (α=0.05) (Zar 1984). As expected, the population size at demographic equilibrium decreased as the extinction rate increased (Table 6). In simulations where the extinction rate was set to zero, the mean population size of 99,998.9 whales was close to the carrying capacity of 100,000 whales. In all simulations where the extinction rate was greater than zero, the population size was depressed below the carrying capacity. When the extinction rate was 1/1000, the mean population size was 98,474.9 whales, and when the extinction rate was increased to 1/100, the mean population size decreased to roughly 84,460 whales, with the standard deviation of the population size being greater in simulated populations experiencing pod extinctions.

Number of pods and pod sizes

The number of pods and pod sizes at demographic equilibrium were determined by the balance between extinctions and splitting events. Two kinds of extinctions occurred during simulations. The extinction rate, x, was set to represent loss of pods due to extrinsic forces such as harvesting, stranding, or disease. Pods could also undergo chance demographic extinction, and the risk of this type of extinction increased as the pod size decreased (Goodman 1987). Because the number of pods remained approximately constant, pods that went extinct were replaced when other pods split.

The mean number of pods in simulations with different parameter combinations were compared using the Tukey multiple comparisons test for unequal

sample sizes (α=0.05). Not surprisingly, the number of pods in the simulated populations changed with the extinction rate and the splitting probability parameter (Table 7). The number of pods increased as the extinction rate decreased or was applied to individuals. When the extinction rate was zero, there were approximately 4510 pods. When the pod extinction rate increased to 1/100, the number of pods decreased to roughly 2018. However, when the extinction rate of 1/100 was applied to individuals, the number of pods was roughly 3706. Changing the splitting probability parameter from 0.7 to zero (x=1/100) decreased the number of pods to approximately 1940.

TABLE 7 : The number of pods, an estimate of the average pod size, and the maximum pod size for simulations with different extinction rates and splitting probability parameters. x is the fraction of pods removed/year, c is the splitting probability parameter, N is the number of simulations with the listed parameter values, "mean" is the mean number of pods for all simulations with the listed parameters (± the standard error of simulation means), "SD" is the standard deviation of the pod number about the mean for values from all simulations with the listed parameters, "ave. size" is an estimate of the average number of whales per pod, and "max. size" is the maximum number of whales in the top observed pod size interval. Pod size distributions were analyzed at years 999, 499,999, and 999,999 for each simulation. All groups in the table have significantly different pod numbers (Tukey test, α = 0.05).

x	c	N	mean (±SE)	SD.	ave. size	max. size
0	0.7	6	4510.47± 0.80	27.68	23	85
1/1000	0.7	2	3766.45± 0.13	28.63	27	85
1/100	0.7	14	2017.59± 0.35	25.28	42	95
1/100(indiv)	0.7	6	3706.13± 0.78	26.09	23	95
1/100	0	2	1940.26± 0.23	23.83	44	95

Pod size distributions were reported in intervals of five whales, and these distributions were examined at years 999, 499,999, and 999,999. The average pod size was estimated by multiplying the midpoint pod size in each interval by the number of pods in that interval and then calculating the average pod size. Increasing the pod extinction rate resulted in larger pod sizes. As the pod extinction rate increased from zero to 1/100, the average pod size increased from 23 whales to 42 whales. However, when the individual extinction rate of 1/100 was used, the average pod size was 23 whales. Changing the splitting probability parameter from 0.7 to zero (x=1/100) increased the average pod size to 44 whales. In all of the sampled pod distributions, the maximum pod size was 85 - 95 whales.

Number of haplotypes per pod

In all of the simulations, most of the pods had only one mtDNA haplotype. The percentage of pods with more than one haplotype was affected by changes in the mutation rate, the extinction rate, the splitting probability parameter, and the starting conditions (Table 8). When the simulations began with the high heterozygosity condition, 8-9% of the pods had more than one haplotype at year 999. However, by year 1999, the numbers of pods with more than one haplotype were comparable to the numbers in the simulations that began with only one haplotype. Consequently, the results below apply to simulations that started with low heterozygosity and simulations that began with high heterozygosity after year 999.

TABLE 8 : The maximum percentage of pods with more than one haplotype for simulations with different parameter values. The reported percentages are for simulations with a low starting heterozygosity and for simulations with a high starting heterozygosity after year 999. u is the mutation rate/individual/year, x is the fraction of pods removed/year, c is the splitting probability parameter, and N is the number of simulations with the listed parameter values. The first parameter values are the standard values, and the parameter value that is changed in the subsequent simulations is highlighted in boldface.

u	x	c	N	maximum %
1.73×10^{-5}	1/100	0.7	10	1.1-1.5%
1.73×10^{-4}	1/100	0.7	2	5-5.6%
1.73×10^{-6}	1/100	0.7	2	0.5-0.7%
1.97×10^{-5}	**1/1000**	0.7	2	0.7-1%
2×10^{-5}	**0**	0.7	6	0.5-0.7%
1.73×10^{-5}	**1/100(indiv)**	0.7	6	0.4-0.5%
1.73×10^{-5}	1/100	**0**	2	2.5-2.7%

When standard conditions were used, fewer than 1.5% of the pods had more than one haplotype. If the mutation rate was increased to 1.73×10^{-4}, the number of haplotypes increased and up to 5.6% of the pods had more than one haplotype. Conversely, if the mutation rate was decreased to 1.73×10^{-6}, the number of haplotypes decreased and fewer than 0.7% of the pods had more than one haplotype. Because the majority of pods in the simulated populations were monotypic, the majority of pods that went extinct were monotypic. Consequently, when extinction rate was decreased to zero or 1/1000, or extinction was set to remove random individuals at the rate of 1/100, fewer monotypic pods were removed and the number of pods with more than one haplotype decreased to less than 1%. When the splitting probability

parameter was set to zero, haplotypes were not sorted during splits and the maximum number of pods with more than one haplotype increased to 2.7%.

DISCUSSION

Genetic Diversity and Pod Extinctions

The observed heterozygosity of 0.14 in North Atlantic pilot whales is unusually low. In other cetacean species that have been studied using mitochondrial D-loop sequencing, including harbour porpoises (*Phocoena phocoena*), common dolphins (*Delphinus delphis*), and bottlenose dolphins (*Tursiops truncatus*), observed heterozygosity ranges from 0.88 to 0.93 (chapter 2, Rosel 1992). This model was designed to address the hypothesis that pod extinctions could have been responsible for low mtDNA diversity in pilot whales. The results of the simulations indicate that if a pilot whale population experienced a moderate level of pod extinctions, it could undergo large fluctuations of mtDNA heterozygosity over time and frequently have heterozygosity as low as that observed in North Atlantic pilot whales.

It must be noted that the large fluctuations in heterozygosity observed under standard conditions (x=1/100, u=1.73x10^{-5}, c=0.7) were not seen when the mutation rate was changed by an order of magnitude or when the pod extinction rate was reduced. When the mutation rate was decreased to 1.73x10^{-6}, heterozygosity in the simulated populations during the last 500,000 years never rose above the value observed in the North Atlantic population. Clearly, if this lower rate results in a more accurate estimation of the true mtDNA mutation rate, pod extinctions certainly

could reduce the heterozygosity in a pilot whale population to the low observed level. However, the heterozygosity in simulated populations with the standard extinction rate and a mutation rate of 1.73×10^{-4} was not reduced to a low level. Consequently, if the standard mutation rate is an underestimate, pod extinctions might not have a significant effect on heterozygosity. In addition, simulations with an extinction rate of 1/1000 indicated that if a pod extinction rate of 1/100 is an unreasonable overestimate, extinction of pods at a more realistic rate might not reduce heterozygosity to the observed low level.

Although the infinite allele model overestimated the heterozygosity in simulated populations with the extinction rate applied to individuals, the heterozygosity in these simulated populations did gradually approach an equilibrium value as predicted, clearly demonstrating that the fluctuations in heterozygosity that occurred with comparable pod extinctions were not caused by the reduction of the population size. Changes in heterozygosity were caused primarily by changes in the relative frequencies of haplotypes rather than the number of haplotypes. Because most pods were monotypic, large numbers of individuals with the same haplotype were removed when entire pods went extinct. Consequently, the relative frequencies of haplotypes could vary significantly over short periods of time when pod extinctions were occurring.

The Effect of the Splitting Probability Parameter on Heterozygosity

It was surprising that reducing the splitting probability parameter to zero increased the amount of time that simulated populations spent with low

heterozygosities. This result might have occurred because reducing c altered the number of pods and pod sizes or because it increased the number of pods with more than one haplotype.

When c=0, each whale had an equal probability of staying or leaving during a split, and large pods were divided into two pods that were approximately the same size. However, when c=0.7, only the leader had an equal probability of staying or leaving, and all other whales would usually follow the leader. Thus, when large pods split during these simulations, the resulting pods were frequently unequal in size because the pods were split based on a smaller sample of unbiased whales. This caused the average pod size in simulated populations where c=0 to be slightly larger than in populations where c=0.7. Consequently, when pods went extinct, a larger number of individuals with the same haplotype were removed from the population.

Reducing c from 0.7 to zero also increased the number of pods with more than one haplotype by a small amount. As a result, pods with more than one haplotype had a slightly greater risk of being removed from the population during simulations when c=0. When new mtDNA haplotypes were created, they would be found in pods with more than one haplotype. Increasing the loss of these pods would have prevented more new haplotypes from having the opportunity to become established in the population.

Assumptions of the Model

This model assumed that mtDNA is completely maternally inherited and that females never move between pods. Takahata and Palumbi (1985) analyzed mtDNA

gene flow in a subdivided population, and their results showed that the mitochondrial genetic differentiation between subpopulations was significantly affected by the effective migration rate. As the effective migration rate increased, the probability that two randomly chosen mtDNA haplotypes sampled from two different subpopulations would be identical increased. The effective migration rate was a function of the relative contributions of male and female mtDNA to succeeding generations and the migration rates of males and females. Under the assumptions of my model, the effective migration rate equals zero. However, if female pilot whales move between pods, the effective migration rate would not be zero. Similarly, if male pilot whales contribute mtDNA to their offspring, the effective migration rate would no longer be zero because males appear to move between pods to mate (Amos et al. 1991a, 1991b, 1993). If the effective migration rate was increased, fewer pods would be monotypic because pods would not be as differentiated. Consequently, large fluctuations in heterozygosity might not be observed under these conditions.

Pod Extinctions in the North Atlantic Pilot Whale Population

The results of this model demonstrate that mtDNA diversity could have been reduced in the North Atlantic pilot whale population because of pod extinctions. If it is assumed that the heterozygosity in this pilot whale population in the absence of pod extinctions would have been similar to the heterozygosities observed other cetacean species, pod extinctions must have reduced heterozygosity from 0.90 to 0.14. During the simulations which had the standard parameter values, reduction of heterozygosity over this range occurred over hundreds of thousands of years.

Pod extinctions could be caused by mass strandings, disease, or harvesting, and the last of these three causes can be attributed to humans. It is unlikely that human exploitation of pilot whales has occurred for hundreds of thousands of years, and if the standard parameter values are good estimates, human exploitation of pilot whale pods could not be solely responsible reducing mtDNA diversity. However, unlike human exploitation, pod extinctions caused by mass strandings and disease have taken place throughout pilot whale history. Long-finned pilot whales are one of the cetacean species that commonly mass strand (Fehring & Wells 1976, Sergeant 1982). Sergeant (1982) estimated that the annual mortality rate of long-finned pilot whales off Newfoundland from 1975 to 1980 due to mass strandings was 1%, and he hypothesized that density-dependent regulation of population size in species that commonly mass strand is through mass strandings. However, it should be mentioned that Tyack et al. (manuscript in preparation) dispute the estimate and conclusions of Sergeant (1962), and they suggest that mass strandings do not significantly contribute to the mortality of species like the pilot whale. The importance of pod extinctions caused by disease is also uncertain. Mass mortality of marine mammals due to disease epidemics has been documented for a number of marine mammals including bottlenose dolphins and many seal species, but not for pilot whales (Harwood & Hall 1990). However, western North Atlantic pilot whale tissue has tested positive for the virus that caused many of the documented epidemics (Patrick Duigan, personal communication).

Importance of the Results for Pilot Whale Management

Even if current human exploitation of pilot whales is contributing to maintaining a low level of mtDNA diversity, it should not be inferred that current exploitation has a significant effect on genetic variation necessary for the health and survival of the species. MtDNA is a neutral genetic marker, and patterns of mtDNA variation may not reflect the patterns of adaptively important genetic variation (Dizon et al. 1992, Lande 1991). In addition, since mtDNA is maternally inherited, if females and males do not have identical dispersal behavior, patterns of nuclear and mitochondrial genetic variation can be very different. Nuclear gene flow can mediated through males even if female movement is limited. In fact, nuclear genetic markers have been effective tools for examining genetic variation and gene flow in eastern North Atlantic pilot whales (Amos et al. 1991a, 1991b, 1993, Andersen 1988). Consequently, for management purposes, it can only be concluded that mtDNA is not an appropriate tool for analyzing patterns of genetic variation in North Atlantic pilot whales.

The Model as a Description of Reality

Any model is limited in its ability to accurately depict reality, and this model was not an exception. Yet in an attempt to make the simulated populations as realistic as possible, many parameters relating to the biology and genetics of pilot whales were included. Incorporating many parameters had the inevitable side effect of creating an almost unlimited number of possible parameter combinations that could have been tested. As a result, many parameters, including those that governed

the population growth rate, the population size, and pod sizes, were held constant, and the three altered parameters were evaluated for only a few possible values. Therefore, the results of this modelling must be considered preliminary. However, the primary goal of the modelling was to determine if pod extinctions could reduce mtDNA diversity in a simplified pilot whale population when critical parameters were set at reasonable values, and the simulations clearly demonstrated that this was possible with certain parameter combinations. The encouraging results indicate that further examination of the effect of pod extinctions on mtDNA diversity in a behaviorally subdivided marine mammal population is worthwhile.

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ACKNOWLEDGEMENT

This model was created in collaboration with Kurt Fristrup, who wrote all of the program code.

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FIGURE 1 : Population growth over time in a simulated population. The probability that an individual will die each year=0.05, and the probability that an individual will give birth each year=0.05[(100,000-N)/100,000]+0.05, where N is the population size.

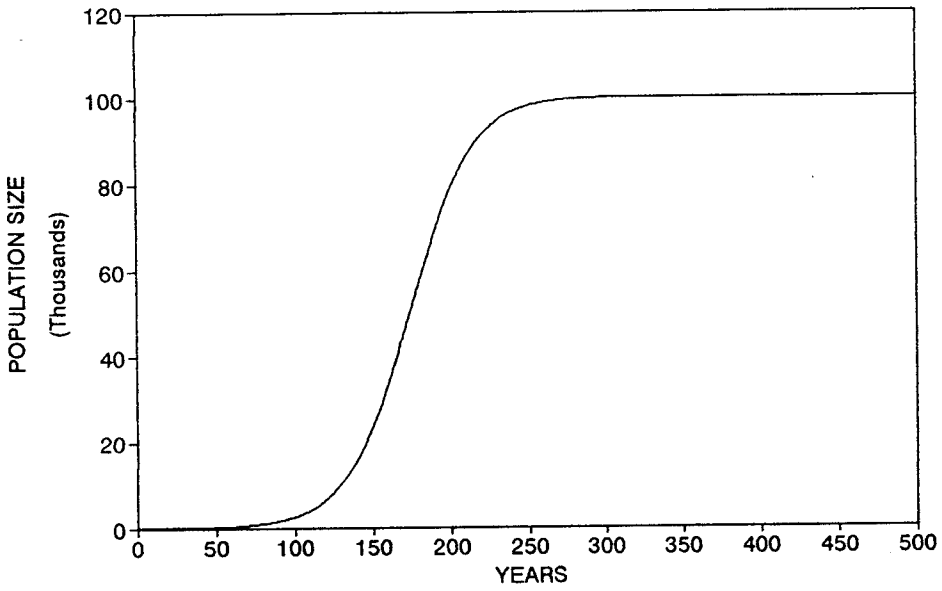


FIGURE 2 : Change in the mutation rate over time in the simulated population of Figure 1 where the mutation rate=$1x10^{-6}$/probability that an individual would give birth each year. When the population size is close to the carrying capacity, the mutation rate=$2x10^{-5}$ mutations/individual/year.

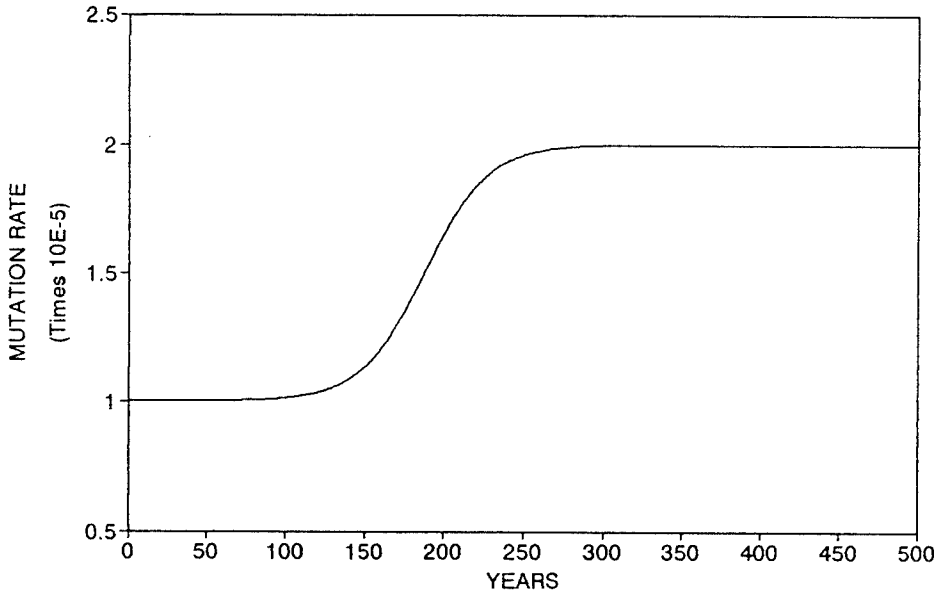


FIGURE 3 : Probability that a pod of size x will split into two pods each year. The sigmoid curve is defined by the equation $[(x/100)^8-(x/100)^4]/[(x/100)^8- 1]$.

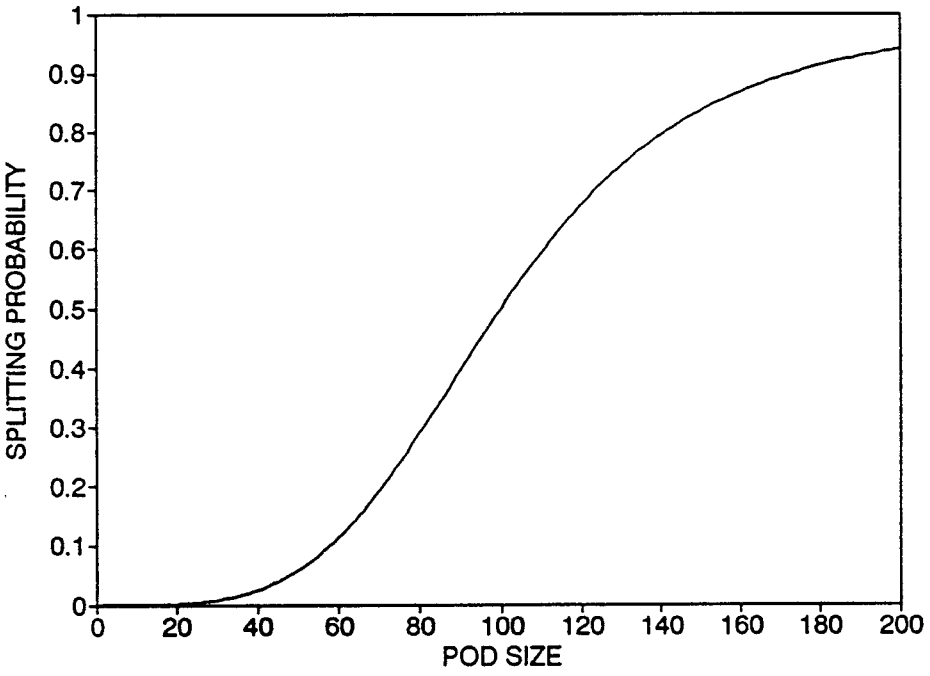
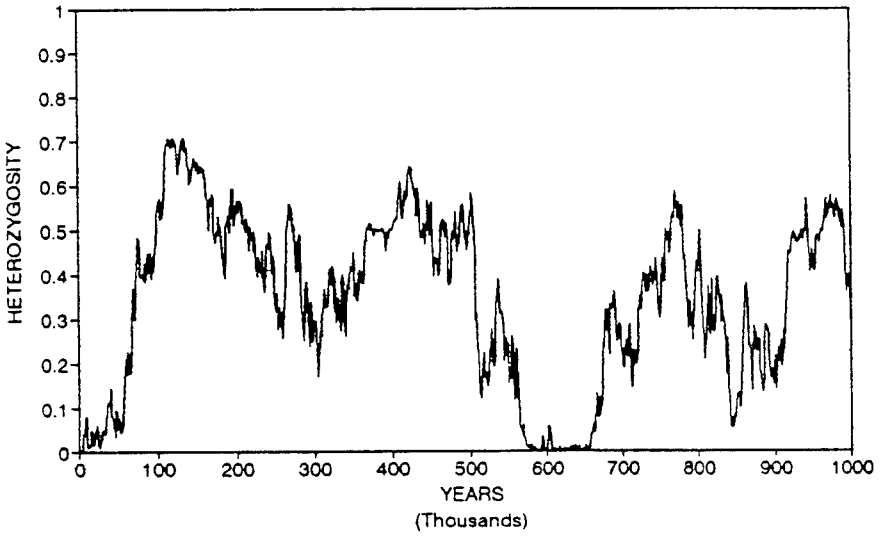


FIGURE 4 : Examples of the changes in heterozygosity over time for two simulated populations that started with low heterozygosity and experienced pod extinctions. The parameter values for both were $u=1.73\text{x}10^{-5}$ mutations/individual/year, $x=1/100$ pod removed/pods/year, and $c=0.7$.



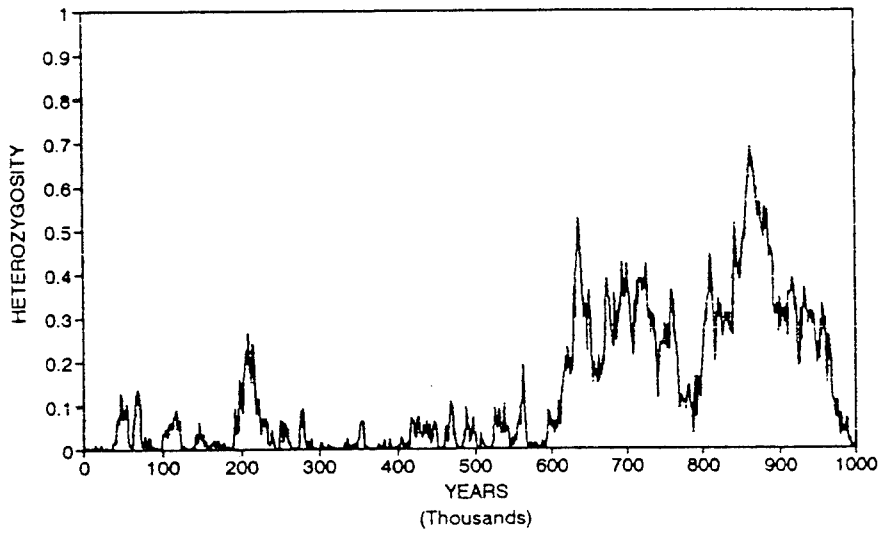
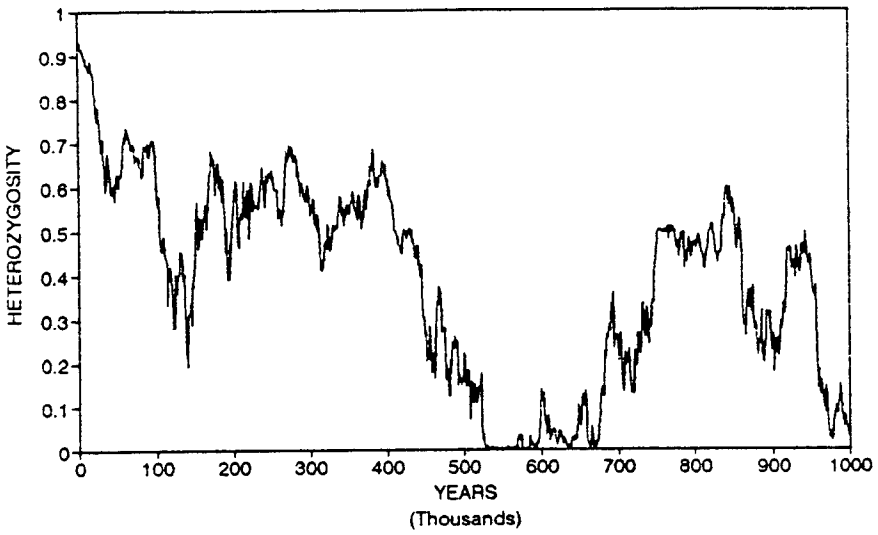


FIGURE 5 : Examples of the changes in heterozygosity over time for two simulated populations that started with high heterozygosity and experienced pod extinctions. The parameter values for both were $u=1.73\text{x}10^{-5}$ mutations/individual/year, $x=1/100$ pod removed/pods/year, and $c=0.7$.



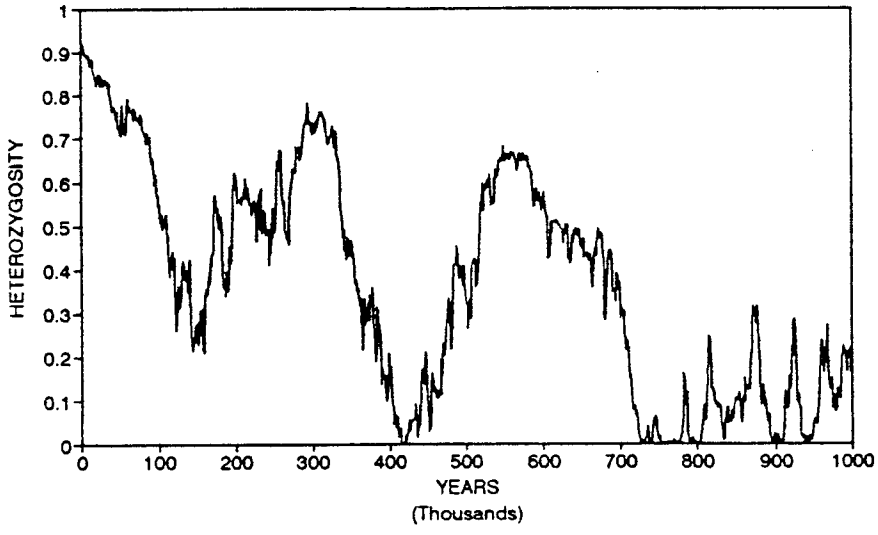
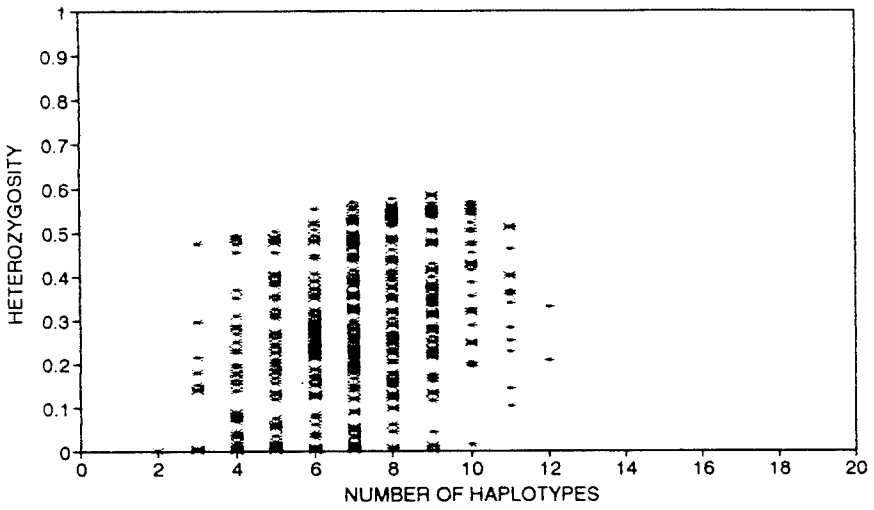


FIGURE 6 : Examples of heterozygosity versus the number of haplotypes during the last 500,000 years of two simulations. The top graph shows the relationship in a simulated population experiencing pod extinctions, and therefore, rapid fluctuations in heterozygosity. The bottom graph shows the relationship in a simulated population experiencing individual extinctions, where the heterozygosity increased over time.



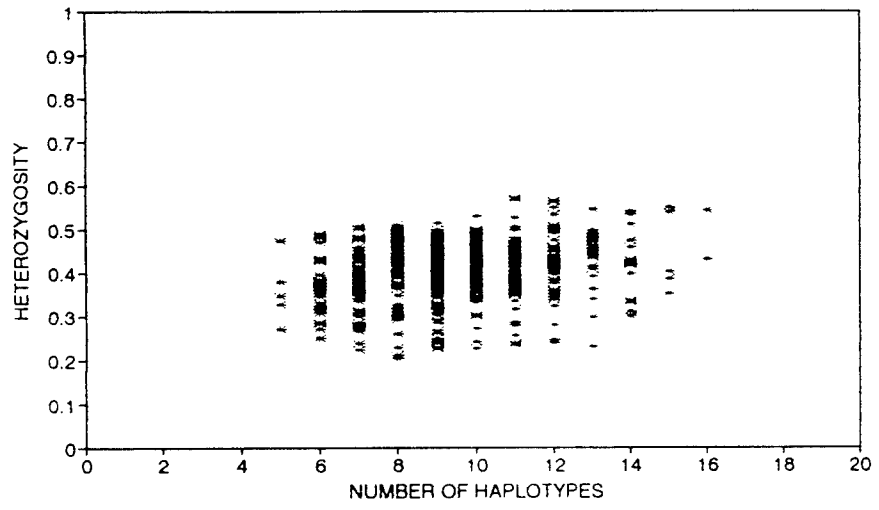


FIGURE 7 : Theoretical and simulated changes in heterozygosity over time for populations that started with heterozygosity=0. The simulated populations experienced only demographic extinctions. The theoretical (smooth) curve was generated using the infinite allele model of Kimura & Crow (1964) with a mutation rate $u=2x10^{-5}$ mutations/individual/year, a population size $N_f=99,999$ whales, and a generation=20 years. The parameter values for the three simulated curves were $u=2x10^{-5}$, $x=0$, and $c=0.7$. The equilibrium heterozygosity under the infinite allele model is 0.799997.

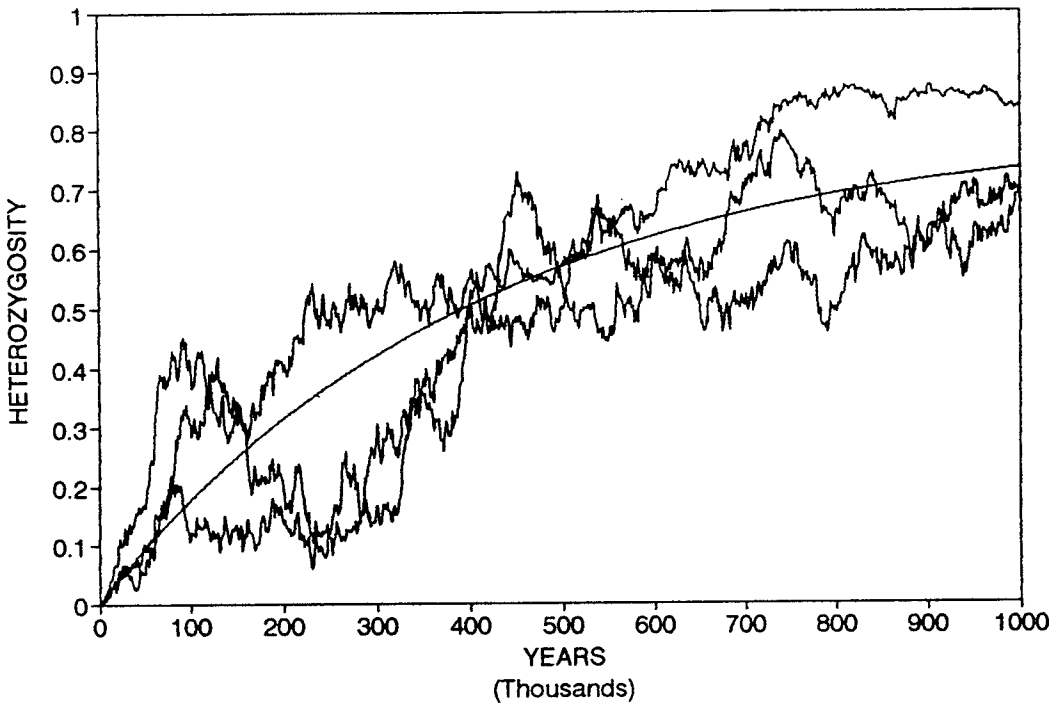


FIGURE 8 : Theoretical and simulated changes in heterozygosity over time for populations that started with heterozygosity=0. The simulated populations experienced demographic extinctions and extinctions of individual whales. The theoretical (smooth) curve was generated using the infinite allele model of Kimura & Crow (1964) with a mutation rate $u=1.73\text{x}10^{-5}$ mutations/individual/year, a population size $N_f=84{,}458$ whales, and a generation=16 years. The parameter values for the three simulated curves were $u=1.73\text{x}10^{-5}$, $x=1/100$ pod removed/pods/year as individuals, and $c=0.7$. The equilibrium heterozygosity under the infinite allele model is 0.745037.

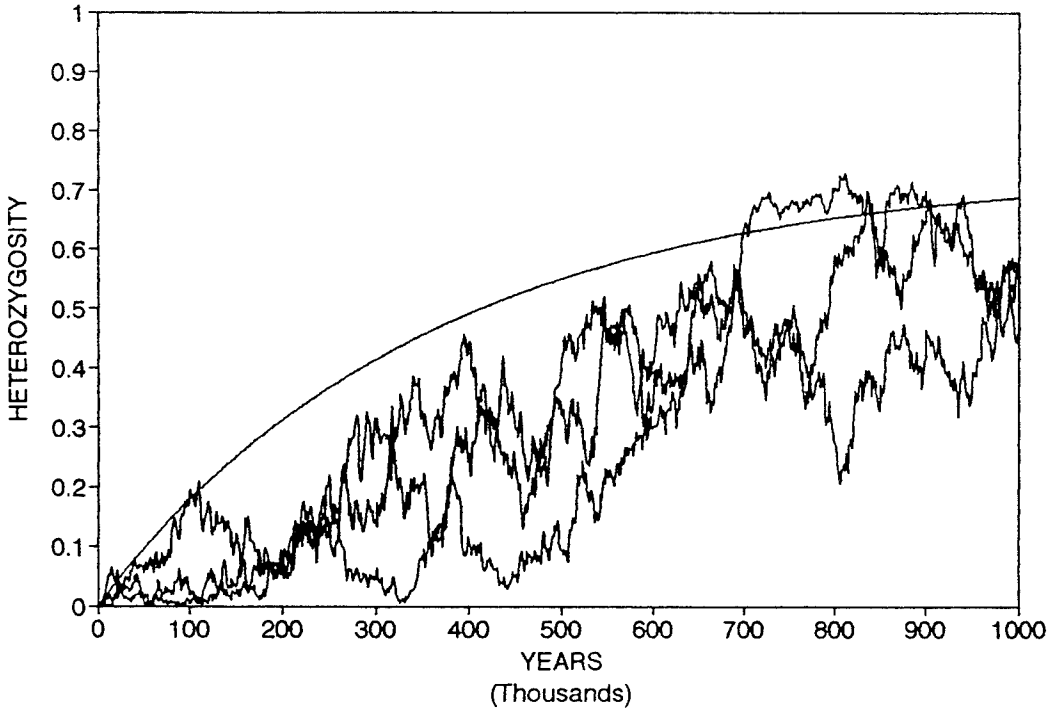


FIGURE 9 : Theoretical and simulated changes in heterozygosity over time for populations that started with heterozygosity=0. The simulated populations experienced demographic and pod extinctions. The theoretical (smooth) curve was generated using the infinite allele model of Kimura & Crow (1964) with a mutation rate $u=1.73\text{x}10^{-5}$ mutations/individual/year, a population size $N_f=84{,}458$ whales, and a generation=16 years. The parameter values for the five simulated curves were $u=1.73\text{x}10^{-5}$, $x=1/100$ pod removed/pods/year, and $c=0.7$. The equilibrium heterozygosity under the infinite allele model is 0.745037.

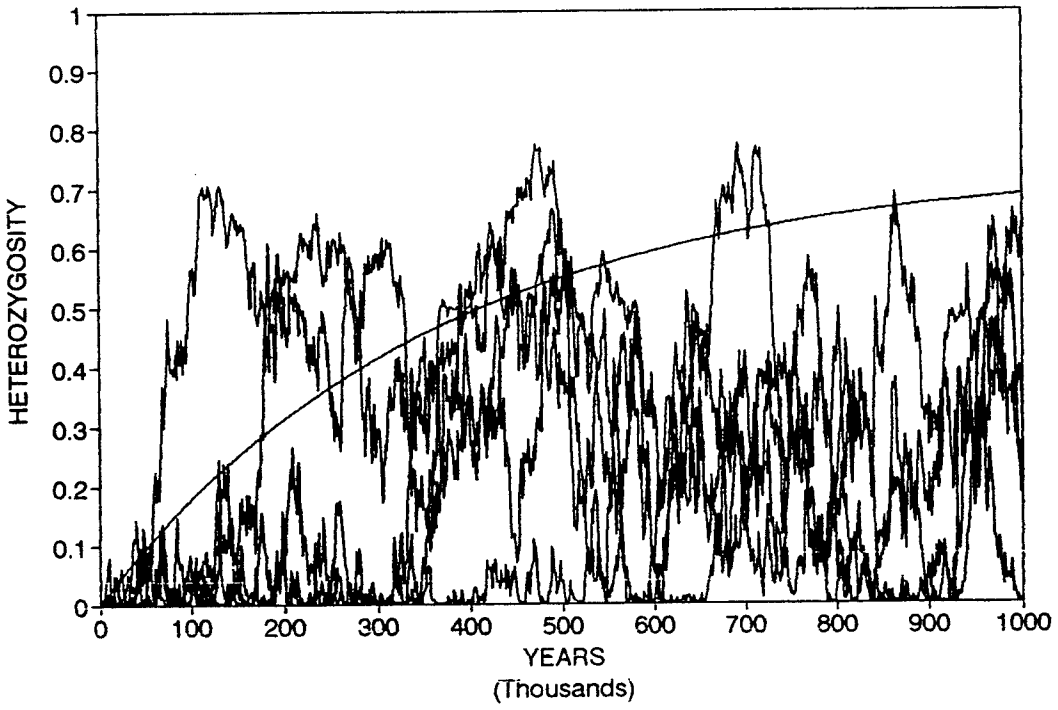


FIGURE 10 : Theoretical and simulated changes in heterozygosity over time for populations that started with heterozygosity=0.95. The simulated populations experienced only demographic extinctions. The theoretical (smooth) curve was generated using the infinite allele model of Kimura & Crow (1964) with a mutation rate $u=2x10^{-5}$ mutations/individual/year, a population size $N_f=99,999$ whales, and a generation=20 years. The parameter values for the three simulated curves were $u=2x10^{-5}$, $x=0$, and $c=0.7$. The equilibrium heterozygosity under the infinite allele model is 0.799997.

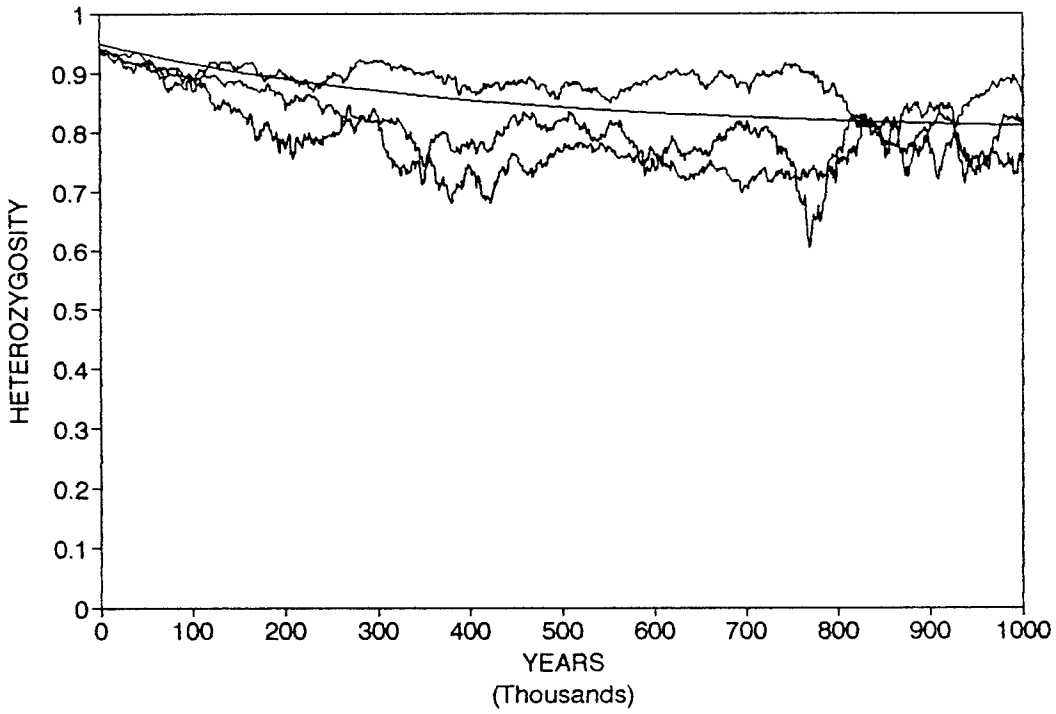


FIGURE 11 : Theoretical and simulated changes in heterozygosity over time for populations that started with heterozygosity=0.95. The simulated populations experienced demographic extinctions and extinctions of individual whales. The theoretical (smooth) curve was generated using the infinite allele model of Kimura & Crow (1964) with a mutation rate $u=1.73\text{x}10^{-5}$ mutations/individual/year, a population size $N_f=84{,}458$ whales, and a generation=16 years. The parameter values for the three simulated curves were $u=1.73\text{x}10^{-5}$, $x=1/100$ pod removed/pods/year as individuals, and $c=0.7$. The equilibrium heterozygosity under the infinite allele model is 0.745037.

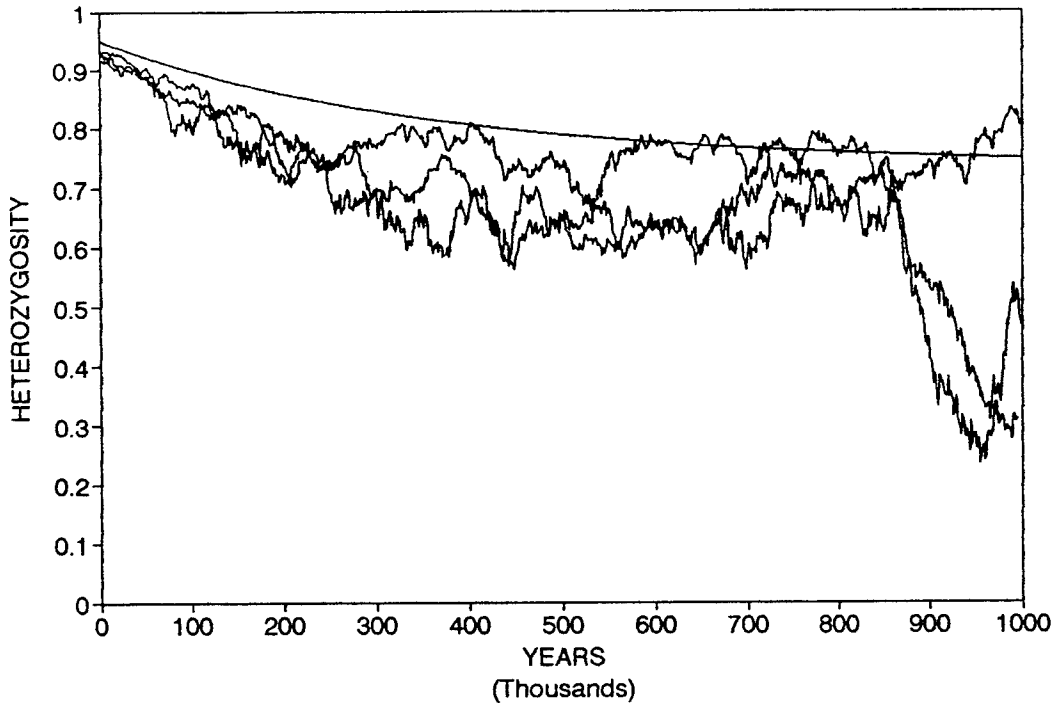
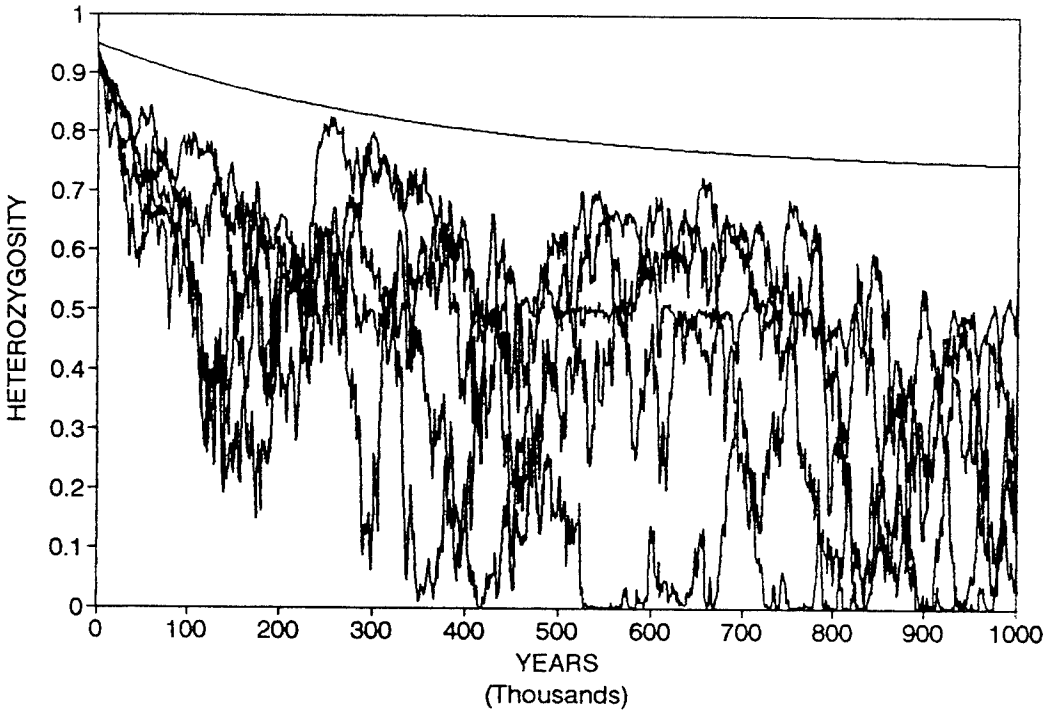


FIGURE 12 : Theoretical and simulated changes in heterozygosity over time for populations that started with heterozygosity=0.95. The simulated populations experienced demographic and pod extinctions. The theoretical (smooth) curve was generated using the infinite allele model of Kimura & Crow (1964) with a mutation rate $u=1.73\text{x}10^{-5}$ mutations/individual/year, a population size $N_f=84{,}458$ whales, and a generation=16 years. The parameter values for the five simulated curves were $u=1.73\text{x}10^{-5}$, $x=1/100$ pod removed/pods/year, and $c=0.7$. The equilibrium heterozygosity under the infinite allele model is 0.745037.



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CHAPTER FOUR

Summary and Conclusions

SUMMARY OF DISSERTATION RESEARCH

Long-finned pilot whales, *Globicephala melas*, are incidentally taken by commercial fisheries operating in US waters (Douglas 1989, Waring et al. 1990) and are or have been hunted in parts of their range (Andersen 1988, Mitchell 1975a, 1975b, Sergeant 1962). To comprehend the impact of these mortalities, it will be necessary to understand the genetic structure of the population. If it is comprised of geographically separated and genetically differentiated subpopulations (genetic stocks), local mortalities could threaten these subpopulations, resulting in the loss of genetic variation necessary for the health and survival of the species. Variation of neutral genetic markers, such as mitochondrial DNA (mtDNA), is one source of information which can be used to define genetic stocks (Dizon et al. 1992, Lande 1991, Ovenden 1990).

My dissertation research focused on answering two questions:

1) Can mitochondrial DNA sequence variation be used to help define genetic stocks of pilot whales in the North Atlantic?

2) Is mtDNA sequence variation affected by the social structure of pilot whales?

I sequenced mitochondrial DNA from 59 whales that stranded on the coasts of Cape Cod, Newfoundland, Nova Scotia, Scotland, and England or were incidentally caught by commercial fisheries operating in the western North Atlantic. Samples from 11 stranded or incidentally caught Atlantic short-finned pilot whales, *Globicephala macrorhynchus*, 2 incidentally caught Pacific short-finned pilot whales, and 11 North Atlantic bottlenose dolphins, *Tursiops truncatus*, were also examined. Mitochondrial DNA sequences were obtained from 400-bp of the D-loop, a non-coding region commonly thought to be the most variable in the mitochondrial genome, and from 303-bp of the protein gene coding for cytochrome *b*.

The D-loop sequences determined from all of the incidentally caught long-finned pilot whales, the long-finned pilot whales that stranded on Cape Cod, the long-finned pilot whales that stranded in Scotland, and the long-finned pilot whales that stranded in England were completely identical. Only the 2 sequences from Canadian whales showed some variability, differing from the other sequence by 0.25 - 0.50% (pairwise sequence divergence). All of the Atlantic short-finned pilot whales had identical D-loop sequences, and this sequence differed from the long-finned pilot whale sequences by 3.25 - 3.75%. The two Pacific short-finned pilot whale sequences differed from each other by 0.25%, from the Atlantic short-finned pilot whale sequence by 0.25 - 0.50%, and from the long-finned pilot whale sequences by 2.75 - 3.50%. D-loop nucleotide diversity in long-finned pilot whales was 0.03% and in short-finned pilot whales was 0.05%. The cytochrome *b* gene sequences determined for 16 long-finned pilot whales from all sampled locations, 4 Atlantic short-finned pilot whales, and two Pacific short finned pilot whales were all identical within each

group, and differed from each other by 0.33 - 0.99%. Finally, D-loop sequences were also determined from 11 bottlenose dolphins. Each dolphin had a distinct D-loop sequence that differed by 0.25 - 4.25% from the sequences of other dolphins, and the nucleotide diversity was 1.25%. Two dolphins were caught together, and the sequence divergence within this pair was 3.50%.

These results suggested that long-finned pilot whales from the eastern and western North Atlantic are not genetically isolated from each other, and that there is only one population of long-finned pilot whales in the North Atlantic. Furthermore, since I observed only three different D-loop sequences in the long-finned pilot whales I surveyed (nucleotide diversity=0.03%), mtDNA variability in North Atlantic long-finned pilot whales may be unusually low. Higher levels of intraspecific D-loop sequence variation have been described in other cetacean species. In addition to the bottlenose dolphin results from this study (nucleotide diversity=1.25%), similar studies on harbour porpoises (*Phocoena phocoena*) and common dolphins (*Delphinus delphis*) found nucleotide diversities ranging from 0.40% for North Atlantic harbour porpoises to 0.78% for short-beaked common dolphins off the coast of California for the same region in the D-loop (Rosel 1992).

It is possible that the low levels of mtDNA variability I observed might be a result of a slow rate of sequence evolution in pilot whales. My sequence analyses showed that there is only a 0.25% sequence divergence between Atlantic and Pacific short-finned pilot whale D-loop sequences (after correction for within-group divergence). This is one sixth of the expected value of 1.5% calculated using a conservative rate of cetacean D-loop evolution of 0.5%/Myr (Hoelzel et al. 1991),

assuming that Atlantic and Pacific short-finned pilot whales have been separated since the closing of the Panamanian land bridge 3 million years ago (Keigwin 1982). Although slow rates of mtDNA sequence evolution have been reported for turtles (Avise et al. 1992, Bowen et al. 1992) and sharks (Martin et al. 1992), such slow rates have not been reported for a mammalian species with a metabolic rate which is at least 5-10 times those of amphibians or fish (Huntley 1987, Lavigne 1982).

As an alternative explanation, I hypothesized that the low level of mtDNA sequence variability I observed was due to metapopulation dynamics resulting from the social system of pilot whales. Mitochondrial gene flow within a pilot whale population is limited due to behavioral constraints. Since mtDNA is maternally inherited, the behavior of females determines mitochondrial gene flow. Pilot whale pods seem to consist of a stable core of related females and their offspring, and unrelated female pilot whales do not appear to join pods (Amos et al. 1991a, 1991b, 1993). Consequently, pods are behaviorally subdivided and mitochondrial gene flow between pods does not occur because females do not move between pods. Genetic variation in a metapopulation, or population of sub-populations, can be lower than predicted using neutral gene theory if the resulting sub-populations are ephemeral (Maruyama & Kimura 1980), and can even approach zero after each sub-population has undergone extinction and been replaced (Gilpin 1991). A pilot whale population can be described as a metapopulation of many sympatric but behaviorally subdivided pods of females, and entire pods can be removed from the population during drive fishery harvesting (Andersen 1988, Mitchell 1975a, 1975b, Sergeant 1962), mass stranding (Sergeant 1982), and disease epidemics (Harwood & Hall 1990). Frequent

extinction of pods, followed by growth and subdivision of surviving pods, could result in the loss of mtDNA variability in a pilot whale population.

This hypothesis is supported by a comparison of mitochondrial genetic variation found in Delphinid species with social systems that are similar to or different from that of pilot whales. Killer whales (*Orcinus orca)*, like pilot whales, have a social system characterized by stable family groups where offspring stay with their mothers (Bigg 1982, Heimlich-Boran 1986), and overall levels of D-loop variability appear to be very low (Hoelzel 1991, Hoelzel & Dover 1991). In contrast, some Delphinid species have social systems characterized by fluid social groups and offspring that do not remain with their mothers after reaching maturity. Examples include bottlenose dolphins, common dolphins (*Delphinus delphis*), and spinner dolphins (*Stenella longirostris*). Studies on these species revealed multiple mitochondrial DNA sequences within populations and levels of mitochondrial variation which were much higher than those found in studies on pilot whales or killer whales (Dizon et al. 1991, Dowling & Brown 1993, Rosel 1992).

To examine the effect of social structure on mtDNA variation in pilot whales more rigorously, I designed a model to study the effect of sub-population extinction on mitochondrial genetic diversity in a pilot whale population which is subdivided as a result of this species' social system. It was an individual-based model that tracked each pilot whale on the basis of her mitochondrial haplotype and pod affiliation. The mutation rate, the extinction rate, and a splitting probability parameter were varied. The standard mutation rate was chosen as a reasonable estimate for the mutation rate per individual per year for a 16,000-bp mtDNA where roughly 9600 basepairs are free

to vary (Palumbi 1989), and the standard extinction rate was chosen because it was within the range of fishery mortality rates reported for drive fisheries in Newfoundland and the Faroe Islands. The standard splitting probability parameter was selected because of the assumption that pilot whales would remain with their closest relatives when a pod split.

The results of the simulations indicated that if a pilot whale population experienced a moderate level of pod extinctions, it could undergo large fluctuations of mtDNA heterozygosity over time and frequently have the low heterozygosity observed in the North Atlantic pilot whale population. In fact, when the mutation rate was set to its standard value, only simulated populations that experienced pod extinctions at a rate of 1/100 pods removed/year frequently spent time with heterozygosity below 0.14, the observed value in North Atlantic pilot whales (27% - 90% of the time during the last half of each simulation). This occurred because changes in heterozygosity were caused primarily by changes in the relative frequencies of haplotypes rather than the number of haplotypes. Because most pods were monotypic, large numbers of individuals with the same haplotype were removed when entire pods went extinct. Consequently, the relative frequencies of haplotypes could vary significantly over short periods of time when pod extinctions were occurring.

Overall, the questions I posed for my dissertation research were answered as follows:

1) Can mitochondrial DNA sequence variation be used to help define genetic stocks of pilot whales in the North Atlantic?

My results indicated that mtDNA is not an appropriate tool for examining the genetic stock structure of North Atlantic pilot whales, since mtDNA variability in these pilot whales is unusually low. This might have been caused by a low rate of mtDNA sequence evolution in pilot whales. In addition, mtDNA variation might not be representative of nuclear DNA variation because female and male pilot whales have different reproductive dispersal behavior.

2) Is mtDNA sequence variation affected by the social structure of pilot whales?

My results confirmed that mtDNA variation can be an indicator of the behavior of females in a species. Because mtDNA is maternally inherited, the behavior of female pilot whales results in the partitioning of mitochondrial genetic variation between pods. The modelling demonstrated that because of this subdivision, pod extinctions could frequently reduce mtDNA diversity in a pilot whale population to the unusually low level I observed using DNA sequencing.

FUTURE DIRECTIONS FOR RESEARCH

1) Examining the genetic stock structure of the North Atlantic pilot whale population

Techniques utilizing nuclear genetic markers such as protein electrophoresis, DNA fingerprinting, and microsatellite analysis have been effective tools for examining genetic variation and gene flow in eastern North Atlantic pilot whales around the Faroe Islands (Amos et al. 1991a, 1991b, 1993, Andersen 1988). Because the reproductive dispersal patterns of male and female pilot whales differ, nuclear gene flow could be mediated through males even if female movement was limited. Consequently, to assess neutral genetic variation in this population of pilot whales, techniques using nuclear markers such as isozymes and microsatellites should be pursued.

2) Examining the social structure of cetacean species

In chapter 2, a limited comparison of genetic studies suggested that mtDNA variability may be consistently lower in Delphinid species with a highly structured social system than in those with a more fluid social system. Consequently, mtDNA surveys might provide a method for obtaining initial evidence concerning the social structure of cetacean species that cannot be easily studied in other ways, particularly if mitochondrial and nuclear genetic markers are used. Further investigation of this possibility, by expanding the list of surveyed species, is needed.

3) Investigating the rate of mitochondrial DNA sequence evolution in pilot whales

The slow rate of mtDNA sequence evolution suggested in chapter 2 was based on a small sample size of 11 Atlantic and 2 Pacific short-finned pilot whales. Clearly, this sample size needs to be increased before it can be stated with any certainty that mtDNA evolves slowly in pilot whales. Further analysis could be pursued using mtDNA sequences from northern and southern hemisphere long-finned pilot whales, although it has been suggested that these antitropical forms may have been separated for only 15,000 years or less (Davies 1962).

4) Continued modelling of mitochondrial genetic variation in pilot whales

The goal of the modelling described in chapter 3 was to determine if pod extinctions could reduce mtDNA diversity in a pilot whale population when other parameters were set at reasonable values. Consequently, the parameter value combinations examined included only a small proportion of possible combinations that could be tested using the model. Aspects of the model which were not studied include the consequences of altering the splitting rule to represent different social structures or changing the demographic parameters. With limited additional programming, the model could also be used to examine the effect of including migration between pods and even the effect of different parameter combinations on simple nuclear genetic variation without recombination.

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REPORT DOCUMENTATION PAGE	1. REPORT NO. WHOI-94-26	2.	3. Recipient's Accession No.

4. Title and Subtitle
Mitochondrial DNA Sequence Variation in North Atlantic Long-finned Pilot Whales, *Globicephala melas*

5. Report Date
June 1994

6.

7. Author(s)
Liese Anne Siemann

8. Performing Organization Rept. No.

9. Performing Organization Name and Address
Woods Hole Oceanographic Institution
Woods Hole, Massachusetts 02543

10. Project/Task/Work Unit No.
WHOI-94-26

11. Contract(C) or Grant(G) No.
(C) NA86-AA-D-SG090
(G)

12. Sponsoring Organization Name and Address
National Oceanic and Atmospheric Administration Sea Grant Program

13. Type of Report & Period Covered
Ph.D. Thesis

14.

15. Supplementary Notes
This thesis should be cited as: Liese Anne Siemann, 1994. Mitochondrial DNA Sequence Variation in North Atlantic Long-finned Pilot Whales, *Globicephala melas*. Ph.D. Thesis. MIT/WHOI, WHOI-94-26.

16. Abstract (Limit: 200 words)

I sequenced mtDNA from 59 long-finned pilot whales (*Globicephala melas*) that stranded on the coasts of Cape Cod, Newfoundland, Nova Scotia, Scotland, and England or were caught by commercial fisheries operating in the western North Atlantic, to determine if there is more than one genetic stock in the North Atlantic. Samples from 13 short-finned pilot whales (*G. macrorhynchus*) and 11 bottlenose dolphins (*Tursiops truncatus*) were also analyzed.

The D-loop sequences determined from 55 of the long-finned pilot whales were completely identical. Nucleotide diversity in long-finned pilot whales was 0.03% and in short-finned pilot whales was 0.05%. These results suggest that long-finned pilot whales from the eastern and western North Atlantic are not genetically isolated from each other and that mtDNA variability in pilot whales may be unusually low. This might be a result of a slow rate of sequence evolution or metapopulation dynamics resulting from the social system of pilot whales.

To examine the effect of social structure, I used an individual-based model in which mtDNA diversity was monitored in a population of pilot whales when extinction rates, mutation rates, and pod dynamics were altered. The results of the simulations indicate that if a pilot whale population experienced a moderate level of pod extinctions, it could undergo large fluctuations of mtDNA heterozygosity over time and frequently have the low heterozygosity observed in the North Atlantic pilot whale population.

17. Document Analysis **a. Descriptors**
pilot whales
mitochondrial DNA
modelling

b. Identifiers/Open-Ended Terms

c. COSATI Field/Group

18. Availability Statement
Approved for publication; distribution unlimited.

19. Security Class (This Report)
UNCLASSIFIED

20. Security Class (This Page)

21. No. of Pages
168

22. Price

(See ANSI-Z39.18) *See Instructions on Reverse*

OPTIONAL FORM 272 (4-77)
(Formerly NTIS-35)
Department of Commerce