MOLECULAR SYSTEMATICS AND POPULATION GENETICS OF MARINE VERTEBRATES FROM BRAZIL

MANUEL ANTONIO DE ANDRADE FURTADO NETO


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

MOLECULAR SYSTEMATICS AND POPULATION GENETICS OF MARINE VERTEBRATES FROM BRAZIL

by

© Manuel Antonio de Andrade Furtado Neto, B.Sc., M.Sc.

A thesis submitted to the School of Graduate
Studies in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Department of Biology
Faculty of Science
Memorial University of Newfoundland

May 1998

St. John's Newfoundland

ABSTRACT

This thesis examines the implications of patterns of DNA sequence variation in a variety of marine vertebrate species of ecological and fisheries interest from Brazilian waters.

The degree of genetic variation in the marine and riverine dolphin *Sotalia fluviatilis* from Brazilian waters was investigated. A unique genotype found only in *Sotalia* from the Amazon River suggests that the freshwater form may be genetically distinct from the marine form. The species is genetically diverse in the marine environment, but the occurrence of a common genotype in all six coastal locations along the marine coast examined suggests that there is sufficient gene flow in the marine region to prevent local differentiation.

A previously unexamined mitochondrial locus, COI (cytochrome oxidase I), was used in combination with three other loci to re-investigate phylogenetic relationships of cetaceans. In this data set, the largest sequence yet applied to this problem, the controversial Milinkovitch Hypothesis that sperm whales are more closely related to baleen whales than to toothed whales was not supported. Instead, four different clades with different taxonomic rankings (Physeteridae, Ziphiidae, Delphinida, and Mysticeti) were identified, in agreement with the traditional separation of toothed and baleen whales as distinct clades. Results of the analysis are sensitive to locus combinations and method of phylogenetic reconstruction.

The species of angel sharks (*Squatina*, Squatinidae) endemic to the continental shelf of Southern Brazil constitute a monophyletic group. The recently described species

S. occulta was found to be more closely related to *S. guggenheim* than to *S. argentina*. This phylogeny helps to explain the evolution of reproductive structures (number of ovaries) and patterns of vertical distribution in the water column (from deep to shallow waters) of squatinid sharks.

Red snapper (*Lutjanus purpureus*) shows high genetic diversity off the coast of northern Brazil. Two genotypic clades have been identified, one of which occurs northwest and the other southeast of the discharge of the Amazon River mouth. This is in agreement with recent morphological and reproductive studies which suggest that *L. purpureus* on the continental shelf of northern Brazil comprises two stock units occupying relatively segregated territories, defined by differences in salinity and temperature.

In contrast, the low genetic diversity of yellowfin tuna (*Thunnus albacares*) from northeastern Brazilian waters agrees with the hypothesis that only a single stock of yellowfin tuna occurs in the southwest equatorial region of the Atlantic Ocean. The genetic homogeneity of *T. albacares* in this area suggests that there is sufficient gene flow in that area to prevent development of local stocks.

To my parents, Araci and Gil,

who always believed in

my potential and supported

all steps of my professional life

over so many years,

and

to my daughters,

Barbara and Nadine.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor, Dr. Steve Carr, who introduced me to the world of molecular genetics and who gave me continued encouragement, friendship, and advice, even during his sabbatical vacation, throughout my PhD program at Memorial University of Newfoundland.

I am very grateful to Dr. Tony Dickinson, member of my Supervisory Committee, who brought me to Canada under a Graduate Scholarship in Marine Science of the Canadian International Development Agency (CIDA), and to Mrs. Colleen Clarke, who always helped with subjects related to my scholarship.

My sincere thanks also go to Dr. Jon Lien, member of my Supervisory Committee and head of the Whale Research Group at the Memorial University, for providing samples of cetaceans for this study, for the interest in my project and for the help in correcting and editing the manuscript.

I would like also to thank the members of my Examination Board, Dr. John Bickham from the Department of Wildlife and Fisheries Science of the Texas A & M University, Dr. Pierre Pepin from the Department of Fisheries and Oceans in St. John's, and Dr. Martin Mulligan from the Department of Biochemistry of the Memorial University of Newfoundland for the suggestions proposed to improve the manuscript.

Many thanks also to my lab mates, Allan Costello, Annette Greenslade, Barbara Saunders, Dave Kivlichan, Dorothy Crutcher, Faye Thompson, John Norman, Michelle Normore, Morgan Vis, Quentin Baldwin, and Shawn Hicks, for the friendly working atmosphere at the Genetics, Evolution and Molecular Systematics Laboratory.

I would like to thank the Biology Department staff, specially Gail Kenny, Peter Earle and Roy Ficken, and the Brazilian students Alberto Campos, Alberto Nunes, Alexandra Magalhães, and Gihad Rabid for the friendship during the years of graduate studies in St. John's.

I also would like to thank Dr. Steven Palumbi, for receiving me at the Pacific Biomedical Kewalo Marine Laboratory of the University of Hawaii and Dr. Úlfur Árnason for receiving me at the Division of Evolutionary Molecular Systematics of the University of Lund, in Sweden. Both of them contributed with helpful comments on the preliminary project of this thesis.

My sincere thanks to Dr. Alexandre Sampaio and Dr. Silvana Saker-Sampaio, for receiving me at the University of Portsmouth, in England, and for the friendship during the last two decades.

Many thanks to Dr. Cassiano Monteiro Neto, the Coordinator of the "Grupo de Estudo de Cetáceos do Ceará (GECC)" at the "Laboratório de Ciências do Mar" of the "Universidade Federal do Ceará", in Fortaleza, Brazil, for accepting the challenge of leading the "Grupo" since the beginning and for always supporting my studies.

I would like to thank all members of the GECC: Ávila, Aline Albuquerque, Aline Martins, Aline Cerqueira, Alexandra Costa, Cristiano, Cristine, Douglas, Guilherme, Juaci, Luísa, Mara, Rebecca, Sérgio Alberto, Tarcísio, and who else I forgot to mention: for the friendship during the many years of work together at the GECC. Special thanks to Francisco Ávila who was very dedicated in collecting samples for this study. "Valeu galera !!!!".

I also would like to thank all the colleagues in Brazil who collaborated collecting samples of dolphins and whales: Alexandre Zerbini, Ana Paula di Beneditto, Eduardo Secchi, Everaldo Queiroz, José Lailson-Brito Jr., José Silva Jr., Marcus Santos, Paulo Flores, Renata Ramos, Salvatori Siciliano, and Dr. Vera da Silva.

Many thanks to Clara Emilie Boeckmann, Otto Gadig, and Marcelo Bezerra for the collection of shark samples, comments in the shark manuscript, and for the friendship and encouragement during the years of this thesis and many previous years. My sincere thanks also to Dr. Solé-Cava for the comments on the manuscript of the sharks chapter.

I would like also to thank Rodrigo de Salles and Dr. Antonio Adauto Fonteles-Filho, for collection of red snappers samples, and Ana Carla El-Deir and Dr. Vera Vieira for the collection of the tunas samples.

Special sincere thanks to Anne Troake for helping to edit most of the text, and for the love and friendship during the last stages of this thesis. Anne will always be in my heart.

Finally, I would like to thanks my parents, Gil Ruben and Araci Tavares de Andrade Furtado and my sister Tania, for all their love, and for their constant emotional, moral and financial support during all stages of my life, including the years during my studies in Canada.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ABI	Applied Biosystems Incorporation
ATP	adenosine triphosphate
bp	base pair
BR	Brazil
CI	consistency index
CIDA	Canadian International Development Agency
COI	cytochrome c oxidase sub-unit one
COII	cytochrome c oxidase sub-unit two
COIII	cytochrome c oxidase sub-unit three
CPUE	capture per unit of effort
CTP	citosine triphosphate
Cytb	cytochrome b
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetate
ESSE	Eyeball Sequence Editor
EU	European Union
GECC	Grupo de Estudo de Cetáceos do Ceará
GTP	guanine triphosphate
HKY	Hasegawa-Kishino-Yano

IUB	International Union of Biochemists
IUCN	International Union for the Conservation of Nature
ICCAT	International Commission for Conservation of the Atlantic Tuna
K2p	Kimura 2-parameters
ML	maximum-likelihood
MP	maximum parsimony
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MUN	Memorial University of Newfoundland
Mya	million of years ago
NADH	nicotinamide adenine dinucleotide de-hydrogenase
NCBI	National Center for Biotechnology Information
NF	Newfoundland
NJ	neighbor-joining
PAUP	Phylogenetic Analysis Using Parsimony
PCR	polymerase chain reaction
PHYLIP	Phylogeny Inference Package
RAPD	randomly amplified polymorphic DNA
REAP	Restriction Enzyme Analysis Package
RI	retention index
RNA	ribonucleic acid
rRNA	ribosomal RNA

Sa-I	sub-area I
Sa-II	sub-area II
Taq	*Thermus aquaticus*
Ti	transition
tRNA	transfer RNA
TTP	thymine triphosphate
Tv	tranversion
UPGMA	Unweighted Pair-Group Method of Arithmetic averages
UV	ultraviolet
WRG	Whale Research Group

CHAPTER 1

GENERAL INTRODUCTION

1.1. Molecular Approach to Systematics

Over the last several decades, biologists from many different fields have turned to genes to study various processes that occur in biological systems. Since the discovery of the molecular basis of inheritance, biomolecules have assumed an enlarged role in evolutionary and population genetics studies and a new science, called molecular systematics, has emerged (Hillis and Moritz 1990; Hillis et al. 1996). Molecular systematics can be defined simply as the study of the diversity of organisms and the relationships among them (Simpson 1961; Wiley 1981) with the use of information from macromolecules.

Three major areas of application of molecular information in systematics were identified by Hillis et al. (1996): (a) Gene evolution, including studies of the processes that generate nucleic acid sequence-level variation, research on the origin of new alleles or new loci, and investigations of convergence and selection; (b) intraspecific or populational studies, including the tracing of organismal and allelic genealogies within species and studies of geographical variation, gene flow, hybridization, and conservation genetics; and (c) interspecific studies, such as the estimation of species phylogenies to evaluate macroevolutionary patterns and processes.

A large and diverse number of applications can be derived from these three areas: ecological and behavioral analyses, developmental studies, investigations of population genetics, and taxonomic and systematics applications (Avise 1994; Hillis et al. 1996).

1.2. Molecules versus Morphology in Systematics

Because of the recent advances in molecular systematics, interest in morphological data as a phylogenetic tool seems to have declined. This is certainly true if one looks at the number of articles on molecular systematics based on deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequences. However, there has been considerable debate over whether molecular or morphological features are better sources of information for estimating phylogeny (Patterson 1987; Hillis 1987; Cracraft and Mindell 1989; Donoghue and Sanderson 1992; Smith and Sytsma 1994; Mishler 1994; Fang el al. 1995; Moritz and Hillis 1996).

The size of the data set available from molecular data is one of the main advantages of molecular systematics (Hillis and Moritz 1990). Each nucleotide position in a nucleic acid sequence can be considered a character, which means that the number of characters available for analysis is immense. On the other hand, the set of morphological data with a genetic basis is a fraction of the molecular information, because all the genetic information of an organism is encoded in its DNA (Hillis 1987). The number of nucleotides in a living organism ranges from 5×10^3 for the smallest viruses, through 13.2×10^6 for bacterial genomes, to 6×10^9 for protist genomes, to

nearly 4×10^{11} for some eukaryotes (Hillis 1987, Avise 1994). Although the potential of molecular information is enormous, real data are generally obtained from a small portion of the entire genome. The analysis of these data may result in cladograms that reflect the evolutionary history of the genes, but perhaps not the evolutionary history of the organisms (Smith and Systma 1994; Moritz and Hills 1996).

Because of the large size and diversity in rates of change in different portions of genomes, biomolecules can provide information about differences between major taxa, e.g. eukaryotes and prokaryotes. In contrast, it is difficult to obtain the same type of information from morphology, because morphological characters are shared among major groups of organisms, eukaryotes *versus* eubacteria, for instance (Hillis 1987).

One advantage of morphological over molecular approaches to systematics has been the routine application of morphological methods to collections of preserved specimens in museums. It is notable that, for many species of poorly known organisms, the only known specimens are represented by the holotype of the species (Hillis 1987). Another advantage of morphological data is that it permits the inclusion of fossil data in phylogenetic analysis. With the development of new techniques (Handt et al. 1994; Höös and Pääbo 1993) it has been possible to sequence DNA from fossil bones of extinct pleistocene fauna (Taylor 1996). Molecular information also has been obtained from traditionally preserved museum specimens (Pääbo et al. 1988; Hagelberg and Clegg 1991; Hagelberg et al 1994), partially cooked meat (Baker et al. 1996), and exotic materials such as whale baleen plates (Kimura et al. 1997)

(extension) (Mullis 1990: Avise 1994: Palumbi 1996). The PCR has become fully automated and can be carried out with commercially available temperature cycles (Avise 1994).

Fragments of DNA produced by PCR amplification can be sequenced directly by manual or by automated sequencing, using an automated DNA sequencer machine (Hillis and Moritz 1990). Automated DNA sequencing is becoming more common as costs decrease. The commercially available automated sequencers can use single-label and four-lane loading separation (Chen 1994). Fluorescent dye-labeled primers, fragments, which are detected by a tunable laser during electrophoresis, are used in this technique rather than radioactively labeled fragments used in manual sequencing.

1.4. Mitochondrial DNA

It is not only the nucleus of cells that contains DNA. Mitochondria and chloroplasts also have DNA molecules that code for all of ribosomal RNA types and for some of the proteins involved in the function of these organelles (Lewin 1997).

In most eukaryotes, mitochodrial DNA (mtDNA) is a small (16,000-18,000 base pairs), double-stranded, circular duplex molecule that replicates semiconservatively and does not interact with chromosomal proteins (Hughes 1990: Klug and Cummings 1994). With some exceptions, animal mtDNA is constituted by a control region and 37 genes, which code for 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs), and 13 messenger RNAs (mRNAs) specifying proteins

Palumbi 1993). These studies showed that the level of amino acid conservation varies significantly among different parts of the cytochrome b molecule (Palumbi 1996). Although cytochrome b is considered 'slow' in terms of amino acid substitutions, the rate of evolution for silent substitutions in third codon positions is similar to that of other mitochondrial genes (Meyer 1994).

Phylogenetic studies of vertebrates using cytochrome b are very common in the literature. Molecular phylogenies of chondrichthyes fishes were proposed by Martin and Palumbi (1993), Martin (1995) and Kitamura et al. (1996) based on the cytochrome b gene. Phylogenetic relationships in teleost fishes, such as tuna (Bartlett and Davidson 1991; Block et al. 1993; Chow and Kishino 1995) and perciform fishes (Cantatore et al. 1994) were also studied using this gene. A molecular phylogeny of mammals was suggested based on cytochrome b by Irwin et al. (1991). Evolutionary relationships in different orders of mammals were also studied using cytochrome b as a molecular marker, such as artiodactyls (Irwin et al. 1991; Graur and Higgins 1994; Honeycutt et al. 1995; Montgelard et al. 1997), carnivores (Arnason et al. 1995; Perry et al. 1995; Ledje and Arnason 1996; Carr and Perry 1997), and cetaceans (Arnason and Gullberg 1994, 1996; Milinkovitch et al. 1994, 1995; Hasegawa et al. 1997).

Cytochrome c oxidase is the terminal enzyme of the electron transport chain and is essential for cell respiration in aerobic organisms (Palumbi 1996; Tsukihara et al. 1996). This oxidase is a dimeric enzyme with each monomer containing three subunits (COI, COII, COIII) encoded by mitochondrial genes and ten encoded by nuclear genes (Cooper et al. 1991; Adkins et al. 1996). The function of cytochrome c oxidase is

related to pumping protons from the matrix side of the mitochondrial membrane toward the intermembrane space (Tsukihara et al. 1996).

The nucleotide sequence of the mitochondrial COI is highly conserved over many taxa (Russo et al. 1996), which makes this gene very useful in phylogenetic reconstruction of deeper evolutionary branches (Palumbi 1996). The COI gene has been successfully used to investigate evolutionary relationships in invertebrates (Bessho et al. 1992; Brown et al. 1994; Hafner et al. 1994; Stern 1994), gadid fishes (Kivlichan 1997), cervines (Carr 1998) and primates (Adkins et al. 1996).

The relative efficiencies of different mitochondrial genes and different tree-building methods in recovering a known vertebrate phylogeny were evaluated by Russo et al. (1996). The genes that produced the truest trees in most tree-building methods or algorithms were the cytochrome b gene and the nicotinamide adenine dinucleotide dehydrogenase subunits 4 and 5 genes (NADH-4 and NADH-5). The COI gene did not produce comparable results, because of the small extent of sequence divergence. The mitochondrial genes that showed the poorest performance were the COII, NADH-1, and NADH-4L (Russo et al. 1996; Russo 1997).

1.6. Molecular systematics and population genetics of marine vertebrates

The use of molecular systematics to study evolutionary relationships among species and population genetics within species of marine vertebrates has increased rapidly in the 1990's. Mitochondrial DNA, random amplified polymorphic DNA

(RAPDs), and microsatellites are now common tools used to investigate evolution and population genetics of sharks, teleost fishes, and marine mammals.

Martin et al. (1992) observed that rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Martin and Palumbi (1993) also investigated the evolution of the mitochondrial cytochrome *b* gene in sharks, and Martin (1995) studied rates, patterns and phylogenetic inference of mtDNA sequence evolution in the same group. Molecular phylogenies for elasmobranch fishes (sharks, skates, rays and chimeras) were proposed by Martin (1995), Dunn and Morrissey (1995) and Kitamura et al. (1996), but none of them included all the orders of elasmobranchs.

Many studies have been performed on evolutionary relationships among bony fishes. Evidence for a slower rate of molecular evolution in teleosts than in mammals was first observed in cytochrome *b* sequences in some perciformes (Cantatore et al. 1994). A molecular phylogeny for scombroids (mackerels, tunas, and billfishes) was proposed by Block et al. (1993) and phylogenetic relationships between tuna species were studied by Chow and Kishino (1995). The population structure of the Atlantic cod fish (*Gadus morhua*) was determined by Carr et al. (1995) based on cytochrome *b* gene sequences, and genetic homogeneity in Greenland halibut (*Reinhardtius hippoglossoides*) in North America was identified using the same mitochondrial gene (Vis et al. 1997).

A molecular view of pinniped relationships with particular emphasis on the true seals (Phocidae) was proposed by Arnason et al. (1995) based on the complete sequences of the mitochondrial cytochrome b gene. Perry et al. (1995) and Carr and

Perry (1997) studied intra- and inter-familial systematic relationships of phocid seals using the same gene.

Population studies in humpback whales have been performed using mitochondrial DNA haplotypes (Baker et al. 1990), nuclear intron sequences and mtDNA (Palumbi and Baker 1994; Baker et al. 1994), and microsatellites (Valsecchi et al. 1997; Palsbøll et al. 1997b). Population structure and dispersal patterns of the beluga whale have been determined in the western Nearctic (O'Corry-Crowe 1997) and in the North Atlantic (Brennin et al. 1997) using mtDNA sequences. Microsatellite markers, which can be used for population studies, were isolated and characterized for beluga whales (Buchanan et al. 1996), sperm whales (Richard et al. 1996), baleen whales (Palsbøll et al. 1997a), bottlenose dolphins (Shinohara et al. 1997), and for some twenty-four species of cetaceans (Valsecchi and Amos 1996).

Molecular phylogenetic relationships among whales, dolphins and porpoises (order Cetacea) have been studied recently by Milinkovitch et al. (1993, 1994, 1995), Arnason and Gullberg (1994, 1996) and Hasegawa et al. (1997). By using myoglobin sequences, mitochondrial ribosomal RNA and Cytb sequences, Milinkovitch et al. (1993, 1994, 1995) suggested that one group of toothed whales, the sperm whales (Physeteridae), is more closely related to the baleen whales (Mysticeti) than to the other toothed whales and dolphins (Odontoceti). Arnason and Gullberg (1994, 1996) challenged the Milinkovitch hypothesis based on complete mtDNA cytochrome b sequences. Hasegawa et al. (1997) evaluated the total molecular evidence for these

hypotheses by applying the maximum likelihood methods to a data set constituted by 12S, 16S, and Cytb mtDNA sequences and myoglobin sequences.

1.7. Science in Brazil

In order to understand the current status of science in Brazil, it is necessary to review some history and place the country in the context of the Latin American scientific community.

Brazil is the largest of the twenty-seven countries of Latin America, with 35% (158.7 million) of the total Latin American population (450 million) in 1990. Although the Latin American population is larger than the United States (U.S.) or the European Union (EU), Latin America's proportion of world's scientific publications was only 1.4% in 1991 compared to 35.8% by the U.S., and 27.7% by the EU (World Scientific Report 1993, Ayala 1995).

The meager scientific production by Latin American countries seems to be a direct consequence of the lack of money invested for scientific research and development. Latin America invested 0.45% of the gross domestic product (GDP) in scientific research and development in 1990, as compared with 2.9% in the U.S. and 2.0% in the EU. Brazil invests, by far, the most money in scientific research and development, a total of 3.179 billion (U.S.$) in 1990, against 961 million (U.S.$) invested in the same year by Mexico, the second largest investor in Latin America (World Scientific Report 1993).

Approximately 35% of the scientific papers from Latin America came from Brazil in 1993. This is more than twice the production of the two runners-up, Mexico and Argentina (about 20%, each) and three times more than Chile (10%). However, the number of publications per 10^6 inhabitants was 26.4 in Brazil, against 62.1 in Argentina, and 19.3 in Mexico (World Science Report 1993). One reason for this low rate was the late start to the process of industrialization in Brazil. Because of this, access to higher education was until recently limited to the upper class. The first Brazilian university was formally founded only in 1920 (da Costa 1995), while Spanish America had 30 universities founded during the colonial period of the 16th and 17th century (Ayala 1995). Before the 1920's, Brazilian higher education was organized in Faculties, not integrated into university campuses. Brazilian universities implemented the first graduate programs only in the 1960's, when they were subjected to major reforms under influence of the military dictatorship (da Costa 1995).

In the 1970's, Brazil had an economic boom mainly because of heavy investments from multinational industries. It was known as the 'decade of growing' when the economy was rigidly controlled by the military government. During this time, research in Brazil was reasonably well-funded and large number of graduate students were sent abroad to train. However, a few scientific fields were favored by this policy, such as strategic areas of physics and engineering, essentially for the nuclear and space programs (da Costa 1995).

After the end of the military dictatorship in 1985, Brazil was immersed in a deep economic crisis which developed during 21 years of military government. The

political transition and the persistence of the economic crisis until the middle 1990's further impeded scientific research as the federal government is the primary source of research funding in Brazil. Because of the crisis, and a focus on short-term financial problems, there was the consequent loss of a long-term development of scientific research.

With the stability of the economy, equality of the Brazilian monetary currency with the U.S.$, and a newly elected president in 1995, there was some real economic growth for the first time in two decades. However, in some scientific fields, the only option to conduct research is to leave the country to study at international facilities. This was true for the field of molecular systematics and population genetics of marine vertebrates.

1.8. Status of molecular systematics and population genetics studies of marine vertebrates in Brazil.

The Brazilian coastline has about 8,500 km, most of them in equatorial and tropical areas. The biodiversity of marine vertebrate species is manifold and many of these species are commercially exploited by fisheries (Fonteles-Filho 1989).

Fisheries in Brazil vary according to geographic regions. In the north and northeast, fisheries are essentially artisanal and only a few resources are extensively exploited. Among these species, the fisheries of spiny lobster (*Panulirus* sp.), red snapper (*Lutjanus purpureus*), and shrimp (*Penaeus* sp.) are the most important. In the south and southeast, fisheries are more industrialized and many species, such as sardines

(Clupeidae), tunas (Scombridae) and sharks (Carcharhinidae), are extensively exploited (Ivo and Sousa 1988; Fonteles-Filho 1989; Salles 1997).

Although total annual fisheries catches in Brazil are between 750.000-1.000.000 metric tons, stock structures of the major exploited species have been little studied and are essentially unknown (Fonteles-Filho 1989). Many species are treated as a single stock, although no scientific data are available to support this assumption. Knowledge of the stock structures of economically important species is essential to the management of fisheries (Fonteles-Filho 1989). To be treated as separate populations for management purposes, fish stocks must be shown to be genetically as well ecologically distinct (Ovenden 1990; Utter 1991; Dizon et al. 1992; Pepin and Carr 1993). Such studies are needed for most commercial species in Brazilian waters.

Some marine vertebrates that were commercially exploited in the past are now symbols of environmental protection in Brazil. Whaling was an intense activity that exploited several species in Brazilian waters until 1987 when it was banned by federal law. Dolphins of all species that occur in Brazilian waters are now also protected, although many species have been taken as fisheries by-catch (Alves-Jr. et al. 1996). The populational structures of whale and dolphin species in Brazilian waters are basically unknown. The identification of these populations and their geographic distributions is a basic priority as a guide to conservation actions.

Molecular systematics is a powerful tool to study evolutionary relationships among and within species, and to investigate population structure specifically by direct analysis of DNA genes sequences (Hillis et al. 1996). Unfortunately, the high

costs of molecular techniques can limit their use in systematics and population genetics. In Brazil, there are few facilities that are able to conduct such type of studies, mainly due to the large initial investment necessary to establish a laboratory and the expense of maintaining the equipment and to keep projects going. None of the appropriately equipped laboratories in Brazil are focused on molecular studies of marine vertebrates.

A number of evolutionary and population studies have been performed at the Genetics, Evolution and Molecular Systematics Laboratory in the Department of Biology at Memorial University of Newfoundland with the use of molecular markers. Genetic and evolutionary relationships in Cervidae species and hybridization between species of North American deer (*Odocoileus*) (Carr and Hughes 1993; Hughes and Carr 1993) were examined using the cytochrome b gene. Population genetics of holartic pine martens (*Martes*) (Hicks and Carr 1991, 1995; Carr and Hicks 1997), and Atlantic cod were also extensively studied using the cytochrome b gene (Carr and Marshall 1991a, 1991b; Pepin and Carr 1993; Carr et al. 1995; Crutcher 1996; Kivlichan 1997).

A PhD program in Biology at the Genetics, Evolution and Molecular Systematics Laboratory of the Memorial University of Newfoundland provided me with the opportunity to investigate several important aspects of evolutionary and population genetics of several important groups of marine vertebrates in coastal Brazil. As a young scientist of the "Laboratório de Ciências do Mar" at "Universidade Federal do Ceará" in Brazil, which has a cooperation program with Memorial University of Newfoundland, my studies in Newfoundland became possible through a

Graduate Scholarship in Marine Science from the Canadian International Development Agency (CIDA).

The primary motivation for the work in this thesis was to investigate the implications of patterns of DNA sequence variation in a variety of species of marine vertebrates of ecological and fisheries interest from Brazilian waters. To my knowledge, this thesis constitutes the first attempt to study molecular systematics and population genetics of marine vertebrates from Brazil using DNA technology.

1.9. Statement of problems and objectives

The species of initial interest in this project was *Sotalia fluviatilis*, a common dolphin along the entire Brazilian coast. In northeastern Brazil, where I come from, this species is a tourist focus, that has been promoted as symbol of ecological tourism by the government of Ceará State, but has also been subjected to fisheries by-catch and some occasional hunting. Based on morphological, behavioral, and ecological variation, two populations or ecotypes, from marine and freshwater environments, have been described (Borobia et al. 1991; da Silva and Best 1994, 1996). However, no molecular data for this species were available. In this thesis work, the first project I undertook was to study the two ecotypes of *S. fluviatilis* in Brazilian waters the genetic variability in mitochondrial DNA sequences (Chapter 2).

As I became aware of the power of molecular techniques, I also became very interested in studying other issues of molecular systematics, such as phylogenetic relationships among cetacean species. A consequence of this interest was the second

thesis project that was to investigate the placement of the sperm whales within cetaceans, which has been debated since different genes or combination of genes have produced different molecular phylogenies for the cetaceans. This study also allowed the construction of molecular phylogenies with the use of DNA sequences of several mitochondrial genes of cetacean species from Brazil and Canada, that were not previously examined. Milinkovitch et al. (1993, 1994) suggested that sperm whales (Physeteridae, Odontoceti) are more closely related to baleen whales (Mysticeti) than to dolphins and toothed whales (Odontoceti). Arnason and Gullberg (1994, 1996) suggested that there are five primary evolutionary lineages of extant cetaceans. These hypotheses were examined with a new mitochondrial gene that has never been used to analyze cetacean phylogeny before (Chapter 3).

With my successive travels to Brazil to collect samples for the two studies mentioned previously, I realized that I could expand my studies to molecular systematics and population genetics of some fish species that are exploited commercially. One group of interest was the angel sharks, which are one of the most important fishery resources from southern Brazil. Phylogenetic relationships of several cryptic species occurring in this area were investigated with the use of mitochondrial DNA sequences (Chapter 4).

Because there is an increasing interest by Brazilian fisheries scientists in using molecular techniques to examine stock structure of important species commercially, I was asked to collaborate in two other projects that involved genetic analyses of red snapper, *Lutjanus purpureus*, from northern Brazil (Chapter 5), and yellowfin tuna,

Thunnus albacares, from the southwest equatorial Atlantic ocean (Chapter 6). Red snapper is the second most important fishery resource in north and northeastern Brazil and tuna fisheries are a growing industry in the same region. The knowledge of the stock structure of these resources is essential for the management of fisheries in northern and northeastern Brazil.

CHAPTER 2

MITOCHONDRIAL DNA SEQUENCE VARIATION IN THE BRAZILIAN DOLPHIN *Sotalia fluviatilis*, FROM COASTAL WATERS

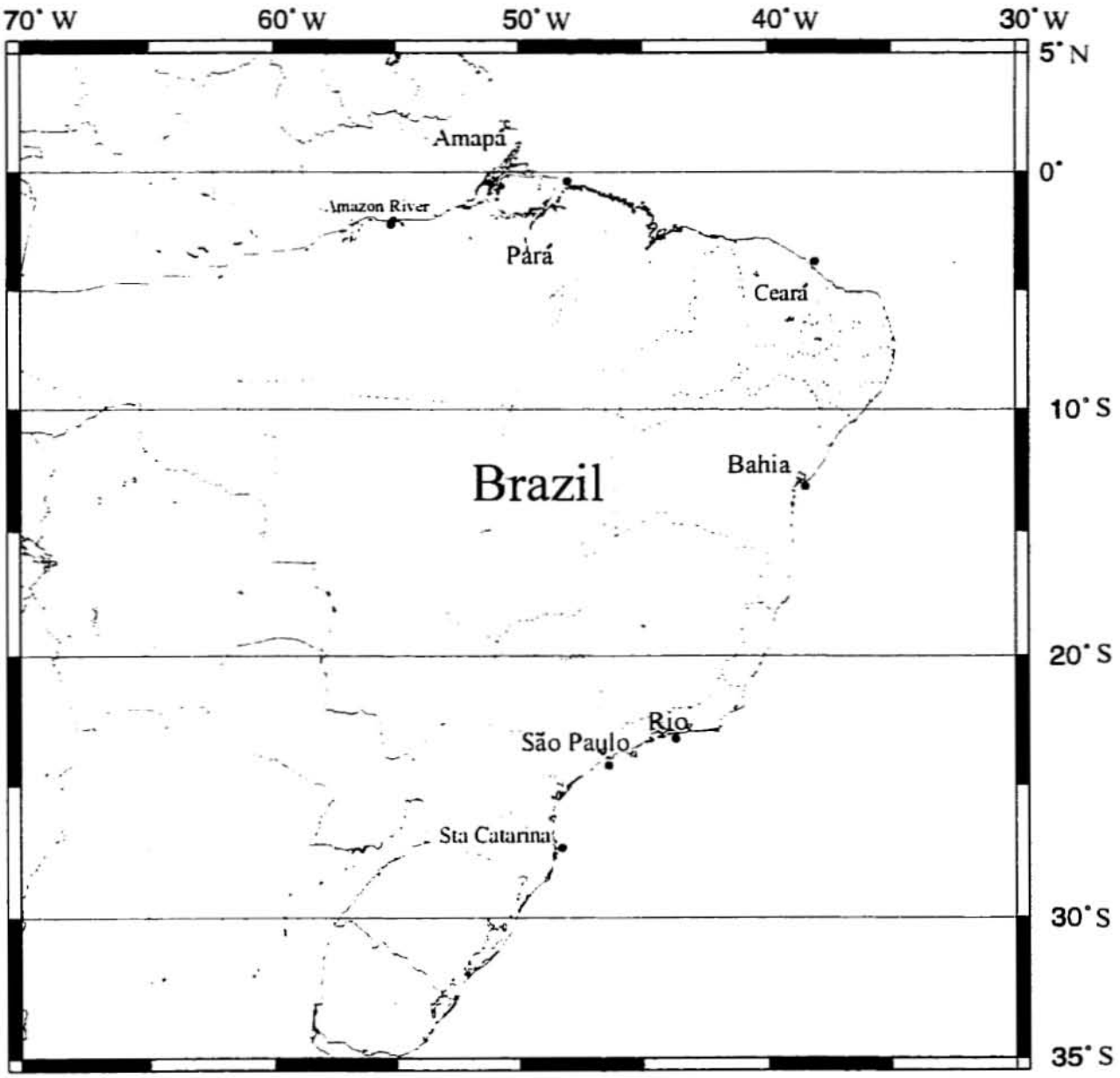
2.1. Introduction

The Brazilian dolphin *Sotalia fluviatilis* (Gervais 1853), known in Portuguese as "boto" or "tucuxi", is the most common dolphin in Brazilian coastal waters with a continuous distribution from Amapá State in northern Brazil (Borobia et al. 1991) to Florianópolis, Santa Catarina State in southern Brazil (Simões-Lopes 1988)(Figure 2.1). In marine waters *S. fluviatilis* occurs along the tropical and sub-tropical Atlantic coast lines of South and Central America, with northern records in Honduras (15°58'N; 85°42'W) and southern limit in Florianópolis (27°35'S; 48°34'W) (Simões-Lopes 1988; da Silva and Best 1996). The species is also found in freshwater in the Orinoco and Amazon River systems (da Silva and Best 1996; Vidal et al. 1997).

The marine and riverine forms were once considered two subspecies, *S. fluviatilis fluviatilis* (Gervais 1853) *and S. fluviatilis guianenses* (van Bénéden 1875) but now are considered two different forms or ecotypes (Borobia et al. 1991; da Silva 1994; da Silva and Best 1994, 1996).

The marine ecotype is larger than the riverine *S. fluviatilis*. The largest known specimens of freshwater *Sotalia* are considerably smaller than the asymptotic length for marine *Sotalia* based on the VonBertalanffy growth model (Borobia 1989). The

Figure 2.1. Map of Brazil, showing the locations (●) and Brazilian States where samples of the dolphin *Sotalia fluviatis* were collected.


70° W
60° W
50° W
40° W
30° W
5° N
0°
10° S
20° S
30° S
35° S
Amapá
Amazon River
Pará
Ceará
Bahia
Brazil
Rio
São Paulo
Sta Catarina

largest recorded marine adults were a 2.06-m female (Barros 1991) and a 2.03-m male (Alves-Jr. et al. 1996), and the largest freshwater adults were a 1.52-m female and a 1.49-m male (Best and da Silva 1984). Borobia (1989) suggested that the differences in size were a sufficient reason for the two forms should be considered separately for management purposes.

The size differences between marine and freshwater ecotypes has been attributed to be a combination of energetic factors and food supply availability. A smaller body size would be advantageous for freshwater *Sotalia*, living in river waters that have almost no temperature changes and are poor in nutrients, particularly rivers with clear waters. On the other hand, a larger body size would be advantageous for marine forms, living in cooler waters in an environment subjected to competition and predation (Borobia 1989; da Silva and Best 1996).

Borobia (1989) investigated 40 morphometric characters of the skulls of marine and freshwater individuals and concluded that differentiation in morphological characters was due solely to differences in size. Differences in size and skull measures were also observed among different locations on the Brazilian coast.

In this chapter, the degree of genetic variation among *S. fluviatilis* from Brazilian coastal waters and between them and one specimen from the Amazon River was investigated with the use of 401-base pair sequences of the mitochondrial cytochrome b gene. A single sample of freshwater *Sotalia* was used in this study due to the difficulty of obtaining more samples from this ecotype.

2.2. Material and Methods

2.2.1. Samples

Tissue (muscle, liver or heart) samples of 30 individuals were obtained from six States in Brazil: three from Pará (including one sample from the Amazon River), twelve from Ceará, two from Bahia, five from Rio de Janeiro, six from São Paulo, and two from Santa Catarina State (Figure 2.1). Samples were collected by the author and colleagues in Brazil.

2.2.2. DNA extraction

DNA was isolated from frozen or DMSO-preserved specimens by an acid guanidium thiosulfate-phenol-chloroform extraction procedure modified from Chomczynski and Sacchi (1987). DNA was extracted with chloroform-isoamyl alcohol (24:1), precipitated with isopropanol, washed with 75% ethanol, and resuspended in 50µl distilled water.

2.2.3. DNA amplification

PCR (polymerase chain reaction) was used to amplify 401-base pair sequences of the mitochondrial DNA cytochrome b gene. The primers used were L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and H15149 (5'-GCCCCTCAGAATGATATTTGTCCTCA-3') (Irwin et al., 1991) for the cytochrome b gene. Each amplification reaction was performed in a 100µl solution containing:

67mM Tris-HCl (pH 9.0). 1.96 mM $MgCl_2$. 9.94 mM β-mercaptoethanol; 2 mM of each dATP. dCTP. dGTP and dTTP; 0.4 μM of each oligonucleotide primer; 1 to 3 units of *Amplitaq* ™ DNA Polymerase (Perkin-Elmer Cetus. Mississauga. ON); and 2 μl of isolated DNA. One drop of light white mineral oil was placed in each tube to prevent evaporation. Amplification was carried out in a Perkin-Elmer Cetus TC-1 Thermal Cycler as follows: initial denaturation at 95°C for 5 minutes. followed by 35 cycles consisting of 93°C for 1 minute (denaturation). 40°C for 1 minute. 55°C for 30 seconds (annealing). 72°C for 2 minutes (extension). and a final step of 72°C for 10 minutes. Electrophoresis of 5μl of PCR product was performed with 1μl dye though 2% NuSieve GTG agarose (FMC Bioproducts. Rockland. ME) gel in 1.0M TBE buffer. PCR products were visualized by staining the gel with ethidium bromide and exposing to ultraviolet (UV) light on an Ultraviolet Transilluminator (Ultra-Violet Products Inc.. San Gabriel. CA) to check if successful amplifications were obtained. Photographs were taken.

2.2.4. Purification of PCR product

PCR product DNA was purified using Wizard™ Magic PCR Preps DNA Purification System (Promega Corp.. Madison. WI) following the manufacturer's instructions. Purified DNA was then quantified with a DNA Fluorometer model TKO 100 (Hoefer Scientific Instruments. San Francisco. CA). Measurement of DNA concentratiom (ng/μl) were obtained using fluorochrome bis-benzimide-zole (Hoechst 33258) which binds to DNA and allows rapid quantification.

2.2.5. DNA Sequencing

The optimum mass of DNA solution was determined by the fluorometer readings (DNA concentration(ng/µl) = 400 / (fluorometer reading x 0.4)) and dried under reduced pressure. Each sample was then resuspended in 7.3 µl of distilled H_2O. 9.5 µl of reaction premix (Applied Biosystems PRISM™ Ready Reaction Dye Deoxy terminator Cycle Sequencing Kit) and 3.2 µl of 1mM primer. The same primers used for PCR were used in separate reactions. Sequencing reactions were carried out in a Perkin-Elmer TC-1 Thermal Cycler in 25 cycles, on the following step-cycle profile: 98°C for 1 second, 50°C for 15 seconds, and 60°C for 4 minutes. Excess primers and unincorporated dye were removed by passing the reaction product through a Sephadex G-50 spin column. The eluted DNA was then dried under reduced pressure and resuspended in 5 µl of a 5:1 mixture of deionized formamide and 50 mM Na_2EDTA (Sigma Chemical Co., St. Louis, MO). Sequencing of both strands of the 401 base pair region was done on an ABI 373A (Applied Biosystems, Inc., Foster City, CA) Automated DNA Sequencer. Samples were loaded into 6% polyacrylamide (19:1 Bis), 7M urea gels, and electrophoresed at 32 watts constant power for 11 hours. DNA sequence data were collected using the ABI collection analysis software package version 1.0.2. Alignments of sequences were done by eye and complementary strands were compared using the Sequence Navigator DNA sequence editor version 1.0.1. (Perkin Elmer, Inc.). Alignment of sequences in a publishable format was obtained from the Eyeball Sequence Editor (ESEE) version 3.0S (Cabot and Beckenbach 1989).

2.2.6. Genetic Heterogeneity and Phylogenetic Analyses

Genetic heterogeneity within samples was estimated by the nucleon diversity (h) index for non-selfing populations and nucleotide diversity (π) index of Nei and Tajima (1981) as calculated by the Restriction Enzyme Analysis Package (REAP) (McElroy et al. 1991) from pairwise divergences calculated by the Phylogenetic Analysis Using Parsimony (PAUP) [version 4.0d61] program of Swofford (1997). The nucleon diversity index is approximately equivalent to the probability that two individuals chosen randomly will have different genotypes. The nucleotide diversity index measures the average pairwise nucleotide difference between individuals within samples, and corrects h for the size of the nucleon examined (Nei 1987; Carr et. al. 1995). Genetic heterogeneity among samples were tested with the Monte Carlo χ^2 test of Roff and Betzen (1989) from REAP: 5000 resamplings of the data matrix were used. Maximum parsimony (heuristic search algorithm, tree-bisection-and-reconnection, with random addition and delayed-character-transformation optimization) networks were obtained by bootstrap analysis for 1,000 replicates, and cluster analyses by the UPGMA and neighbor joining algorithms were performed with PAUP.

2.3. RESULTS

Within the 401-bp region examined, seven variable sites were identified among the 30 dolphins sampled (Figure 2.2). Six substitutions occur at the third positions in their codons. Of these, four are pyrimidine transitions and two are purine transitions. The other substitution occurs at first position and is a silent leucine codon pyrimidine transition. The variable nucleotide sites identified here define six genotypes that differ by one to four nucleotide substitutions. Although a single sample was available from the Amazon River, the genotype of this sample was detected to be distinct at least three nucleotides differences from the other genotypes (Figure 2.3).

Genotype A, the most common genotype, is found in samples from all locations except in the single sample from the Amazon River. Genotype B was identified in Ceará and Rio de Janeiro, genotype C only in Ceará, genotype D only in Bahia, genotype E in São Paulo and Santa Catarina, and genotype F only in the Amazon River sample (Table 2.1).

The Monte Carlo χ^2 test indicates significant differences of genotype distributions among the seven samples ($\chi^2 = 57.76$, $df = 30$, $p < 0.05$). However, there are no significant differences if the single Amazon River sample is removed from the analysis ($\chi^2 = 26.84$, $df = 25$, $p > 0.05$), or if dolphins from the northern (Pará, Ceará, Bahia) and southern States (Rio de Janeiro, São Paulo, Santa Catarina) are pooled separately and compared (northern *versus* southern, $\chi^2 = 5.13$, $df = 5$, $p > 0.05$).

The nucleon diversity (h) and nucleotide diversity (π) indices within samples are given in Table 2.2. The probability that any two dolphins chosen at random will

have different genotypes is about 40% (mean nucleon diversity = 0.37). Among the current samples, those from Bahia and Santa Catarina have the highest nucleon diversity. The mean value of 0.0012 for the nucleotide diversity indicates that any two dolphins chosen at random differ on average by about 0.5 nucleotide in the 401-bp region examined.

Nucleotide divergences between samples from different locations are given in Table 2.3. This calculation includes a correction for nucleotide diversity within samples from the same location: a negative value indicates that the average within-sample variation is greater than between-sample difference (Carr et al. 1995). This is the case in 4 of the 21 pairwise comparisions.

Both UPGMA and neighbor joining analyses (trees not shown) of nucleotide divergence distance indicate that the genetic differences that exist among *S. fluviatilis* from coastal waters are not related to their geographic distribution in the marine environment. However, the sample from Amazon River was detected to be genetically distant from the others by both analyses.

Figure 2.2. Variation in DNA sequence of six genotypes of *Sotalia fluviatilis* within a 401-bp region of the mitochondrial cytochrome b gene. Dots represent nucleotides that are identical to that in the genotype A. The top line gives the inferred amino acid sequence according to the single letter code of the International Union of Biochemists. Numbers at the end of the first and second line indicate the position numbers in the protein and nucleotide sequences, respectively.

```
            M   T   N   I   R   K   T   H   P   L   M   K   12
Sotalia-A   atg acc aac atc cga aaa aca cac cca cta ata aaa  36
Sotalia-B   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ... ... ...

            I   L   N   N   A   F   I   D   L   P   T   P   24
Sotalia-A   atc ctc aat aac gca ttc att gac cta ccc act cca  72
Sotalia-B   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ..c ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ..t ... ...

            S   S   I   S   S   W   W   N   F   G   S   L   36
Sotalia-A   tcc agc atc tcc tca tga tga aat ttt ggt tcc cta  108
Sotalia-B   ... ..t ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ..g ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ... ... t..

            L   G   L   C   L   I   M   Q   I   L   T   G   48
Sotalia-A   cta ggc ctc tgc cta att ata caa atc cta aca ggt  144
Sotalia-B   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ... ... ...

            L   F   L   A   M   H   Y   T   P   D   T   S   60
Sotalia-A   tta ttt cta gca ata cac tac aca cca gac acc tca  180
Sotalia-B   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ... ... ...

            T   A   F   S   S   V   A   H   I   C   R   D   72
Sotalia-A   act gct ttt tca tca gtc gca cac atc tgt cga gac  216
Sotalia-B   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ..c ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ... ... ...

            V   N   Y   G   W   F   I   R   Y   L   H   A   84
Sotalia-A   gtc aac tat ggc tga ttc atc cgc tat tta cat gca  252
Sotalia-B   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E   ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F   ... ... ... ... ... ... ... ... ... ... ... ...
```

```
             N   G   A   S   M   F   F   I   C   L   Y   A    96
Sotalia-A  aac gga gct tcc ata ttc ttc atc tgc ctt tac gcc   288
Sotalia-B  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F  ... ... ... ... ... ... ... ... ... ... ... ...

             H   I   G   R   G   L   Y   Y   G   S   Y   M   108
Sotalia-A  cac atc gga cgt ggc cta tac tat ggc tct tat ata   324
Sotalia-B  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F  ... ... ..g ... ... ... ... ... ... ... ... ...

             F   Q   E   T   W   N   I   G   V   L   L   L   120
Sotalia-A  ttc caa gaa aca tga aac att ggc gta ctc ctc cta   360
Sotalia-B  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F  ... ... ... ... ... ... ... ... ... ... ... ...

             L   T   V   M   A   T   A   F   V   G   Y   V   132
Sotalia-A  cta aca gtc ata gcc act gca ttc gta ggt tac gtc   396
Sotalia-B  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-C  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-D  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-E  ... ... ... ... ... ... ... ... ... ... ... ...
Sotalia-F  ... ... ... ... ... ... ... ... ... ... ... ...

             L                                             133
Sotalia-A  cta cc                                          401
Sotalia-B  ... ..
Sotalia-C  ... ..
Sotalia-D  ... ..
Sotalia-E  ... ..
Sotalia-F  ... ..
```

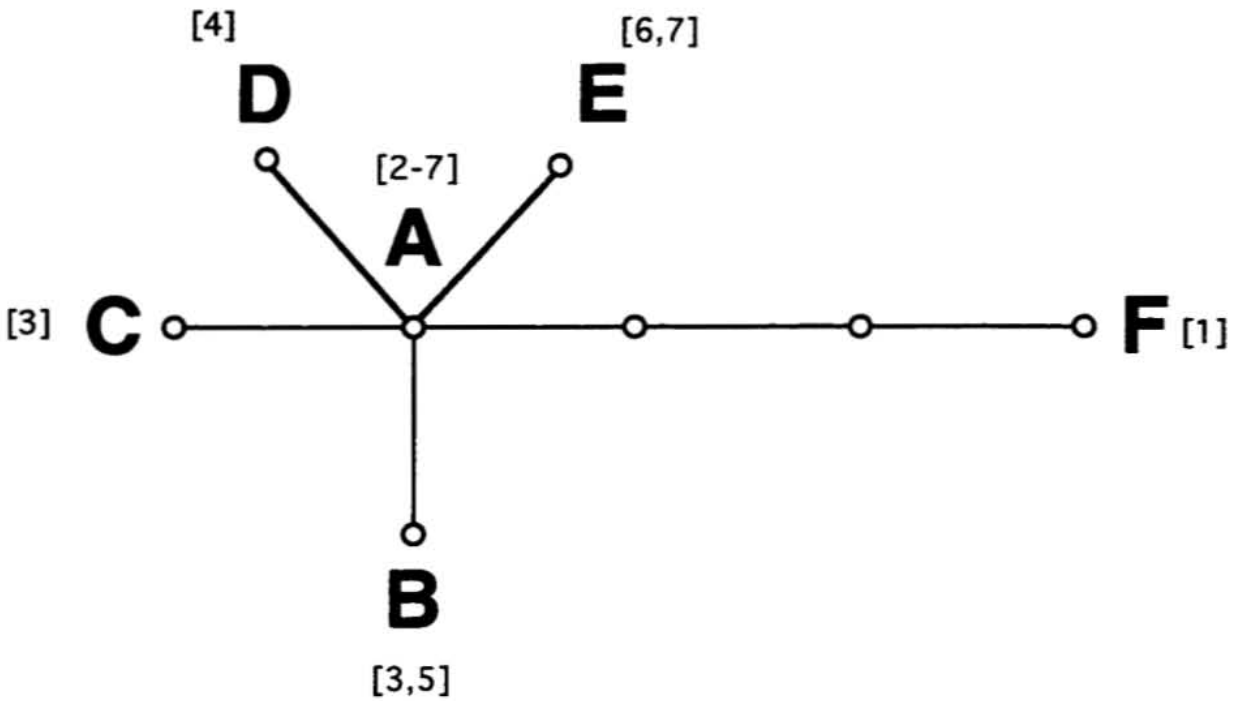


Figure 2.3. Phylogenetic relationships of six genotypes among seven sample locations of *Sotalia fluviatilis*. A maximum parsimony network is shown, where each branch represents a single nucleotide substitution. Numbers in brackets indicate locations in which that genotype was found: 1=Amazonas; 2=Pará; 3=Ceará; 4=Bahia; 5=Rio de Janeiro; 6=São Paulo; 7=Sta. Catarina.

Table 2.1. Distribution of mtDNA genotypes of *Sotalia fluviatilis* among seven sampling locations in Brazilian waters.

		mtDNA Genotype					
Locality	n	A	B	C	D	E	F
Amazonas	1	0	0	0	0	0	1
Pará	2	2	0	0	0	0	0
Ceará	12	8	2	2	0	0	0
Bahia	2	1	0	0	1	0	0
Rio de Janeiro	5	4	1	0	0	0	0
São Paulo	6	5	0	0	0	1	0
Sta. Catarina	2	1	0	0	0	1	0
Total	30	21	3	2	1	2	1

Table 2.2. Haplotype (h) and nucleotide (π) diversity indices within samples of *Sotalia fluviatilis* from seven sampling locations from Brazil.

Locality	Haplotype diversity	Nucleotide diversity
Amazonas	0.0199	0.000 200
Pará	0.0392	0.000 099
Ceará	0.5217	0.001 510
Bahia	0.6667	0.002 400
Rio de Janeiro	0.3556	0.000 996
São Paulo	0.3030	0.000 830
Sta. Catarina	0.6667	0.002 490
Mean	0.3675 ± 0.010 370	0.001 218

Table 2.3. Nucleotide divergences among samples of *Sotalia fluviatilis* from seven locations in Brazil.

	Amazonas	Pará	Ceará	Bahia	Rio de Janeiro	São Paulo	Sta. Catarina
Amazonas	---						
Pará	0.009 780 399	---					
Ceará	0.009 855 551	0.000 058 747	---				
Bahia	0.009 824 950	0.000 000 989	0.000 091 818	---			
Rio de Janeiro	0.009 780 400	-0.000 009 842	-0.000 090 515	0.000 010 000	---		
São Paulo	0.009 780 400	0.000 000 032	0.000 075 707	0.000 008 333	0.000 000 033	---	
Sta. Catarina	0.009 780 400	0.000 000 098	0.000 076 818	0.000 025 000	-0.000 001 000	-0.000 415 000	---

2.4. DISCUSSION

Analysis of mitochondrial DNA sequence variation in *S. fluviatilis* identified a high degree of polymorphism (seven genotypes in 30 samples) and genetic diversity (global $h = 0.37$; global $\pi = 0.0012$). A common genotype was present in all marine locations. Genotype proportions were significantly differentiated amongst sample populations when the Amazon River sample is included, but not when this sample is excluded. Phylogenetic analyses of the seven genotypes identified a difference between the single available Amazon River sample and the marine samples, but did not provide any indication that the genetic variation among marine *S. fluviatilis* is subdivided among geographic samples (Figure 2.3).

These results suggest that the freshwater form of *S. fluviatilis* may be genetically distinct from the marine form. This is in agreement with previous studies, based on meristic and morphometric characters, that have identified distinguishable ecotypes of *S. fluviatilis* from freshwater and marine water (Borobia 1989; Borobia et al. 1991).

The presence of five different genotypes of *S. fluviatilis* in the coastal waters of Brazil indicates that the species is genetically diverse in the area studied. The occurrence of a common genotype in all six locations along the marine coast suggests that there is sufficient gene flow in the marine region to prevent high genetic differentiation.

The presence of the common genotype A in the mouth of the Amazon River (Pará) suggests that individuals of the marine ecotype can live in conditions of low

CHAPTER 3

MOLECULAR PHYLOGENY OF CETACEANS AS SUGGESTED BY THE CYTOCHROME OXIDASE I GENE ALONE AND IN COMBINATION WITH OTHER MITOCHONDRIAL GENES

3.1. Introduction

Whales, dolphins and porpoises (order Cetacea) are aquatic mammals that are among the most specialized of all living organisms. They inhabit all the oceans and some species also live in river systems. According to traditional classification, the order Cetacea includes three suborders: the extinct suborder Archaeoceti known only from fossil records; and the extant suborders Mysticeti, the filter-feeding baleen whales, and Odontoceti, which includes the toothed whales, dolphins and porpoises (Jefferson et al. 1993).

The main morphological difference between mysticetes and odontocetes that has been used to separate these two groups is the presence of baleen in the mysticetes and teeth in the odontocetes. The presence of baleen has been interpreted as a synapomorphy (a shared derived character state: Hennig 1966) only found in extant mysticetes (some extinct mysticete taxa possesed teeth and may not have had baleen (Heyning 1997)), while the presence of teeth has been regarded as a symplesiomorphy (a shared ancestral character state: Hennig 1966) because other mammals have teeth (Milinkovitch 1995).

Another difference is that odontocetes also have the ability to echolocate, with pulses of high frequency sound that are used to explore the environment and search for prey (IUCN 1991). According to Milinkovitch (1995) that ability was probably present in the ancestor of all cetaceans, since baleen whales have a "vestigial melon", one of the main components of the echolocation system. Echolocation in Odontoceti is considered to be a symplesiomorphy of Cetacea.

Van Valen (1966) proposed that cetaceans arose from primitive condylarth mesonychids, an extinct group of ungulates, in the middle or late Paleocene. Recently paleontological findings (Gingerich et al. 1990, 1994; Thewissen and Hussain 1993; Thewissen et al. 1994) and analysis of mitochondrial gene sequences also have suggested that the ungulates are the terrestrial mammals most closely related to cetaceans (Arnason et al. 1991; Irwin et al. 1991; Douzery 1993; Milinkovitch et al. 1993, 1994; Cao et al. 1994; Graur and Higgins 1994; Irwin and Arnason 1994; Arnason and Gullberg 1994, 1996; Montgelard et al. 1997). Artiodactlys were indicated to be more closely related to cetaceans than to perissodactyl ungulates (Czelusniak et al. 1990; Gingerich et al. 1990; Irwin et al. 1991; Milinkovitch et al. 1993). Based on mitochondrial and nuclear gene sequence data the semi-aquatic hippopotamid artiodactyls were proposed to be the closest extant relatives of cetaceans (Irwin and Arnason, 1994; Gatesy et al., 1996; Arnason and Gullberg, 1996; Gatesy, 1997), but this hypothesis has been challenged by Hasegawa and Adachi (1996). Based on combined analysis of complete mitochondrial cytochrome b and 12S rRNA sequences of 17 representatives of Artiodactyla and Cetacea a monophyletic

Cetacea+Artiodactyla clade (defined as "Cetartiodactyla") was proposed by Montgelard et al. (1997).

Based on paleontological (Van Valen 1968; Barnes et al. 1985), chromosomal (Arnason 1972, 1974, 1982; Kulu 1972) and molecular studies (Milinkovitch et al. 1993, 1994, 1995; Arnason and Gullberg 1994, 1996; Montgelard et al. 1997; Gatesy 1997; Hasegawa et al. 1997), cetaceans have been considered to constitute a monophyletic group. However, the fossil record is incomplete and the relationships among the Archaeoceti and the two extant suborders are not well established (Barnes 1984; Barnes et al. 1985; Milinkovitch et al. 1994). The oldest supposed cetacean, *Pakicetus inachus*, is a 52 million-year-old fossil collected in Pakistan (Thewissen and Hussain 1993; Thewissen et al. 1994). It has been suggested that extant cetaceans separated from the extinct archaeocetes 35–45 million years ago (Barnes et al. 1985; Fordyce 1992; McLeod et al. 1993), but there is no clear evidence if the archaeocetes gave rise to one, both, or neither suborder of living cetaceans (Milinkovitch et al. 1995).

Recently, a controversial hypothesis based on molecular phylogenetic analyses suggested a sister relationship between sperm whales (sub-order Odontoceti: superfamily Physeteroidea) and baleen whales (sub-order Mysticeti). This hypothesis was first suggested based on an analysis of myoglobin amino acid sequences of ten cetacean species, and on an analysis of partial sequence data (930bp) of mitochondrial 12S and 16S ribosomal genes of 16 cetacean species (Milinkovitch et al. 1993), and later, based on combined partial sequence data (1,532bp) of the same two ribosomal

genes and partial cytochrome b gene sequences of 21 species representative of all major groups of cetaceans (Milinkovitch et al. 1994). The suggested paraphyly of toothed whales contrasted sharply with the traditional separation of cetaceans in sub-orders Odontoceti and Mysticeti. It also implied, on the assumption that the molecular divergence rates of cetaceans and ungulates are similar (Kraus and Miyamoto 1991; Arnason et al. 1991; Allard et al. 1992), that the common ancestor of sperm whales and baleen whales lived only 10-15 million years ago instead of 30-45 million years as previously believed (Barnes et al. 1985; Milinkovitch et al. 1993).

However, phylogenetic analyses based on the complete sequence of the mitochondrial cytochrome b gene of fourteen cetacean species (Arnason and Gullberg 1994), and 28 species from all thirteen currently recognized families (Arnason and Gullberg 1996) did not identify a close relationship between sperm whales and baleen whales. These authors claimed to have identified five primary evolutionary lineages of extant cetaceans, one represented by Mysticeti and four represented by the odontocete superfamilies Platanistoidea, Physeteroidea, Ziphioidea, and Delphinoidea. Arnason and Gullberg (1996) observed that their phylogeny was sensitive to ingroup representation and the choice of outgroup. Cytochrome b relationships among baleen whales, sperm whales, and dolphins were also found to be sensitive to character weighting, to species sampling, and to choice of outgroup (Adachi and Hasegawa 1995; Milinkovitch et al. 1996).

Hasegawa et al. (1997) used combined data from the mitochondrial 12S and 16S rRNA, cytochrome b, and nuclear myoglobin genes to study the relationships

among the major groups of cetaceans. They concluded that, although the placement of Ziphioidea remains uncertain, their analyses strongly suggested that the toothed-whale monophyly should be revised, as was proposed by Milinkovitch et al. (1993, 1994).

A recent cladistical analysis based on 75 morphological characters from species of all families of extant cetaceans and some fossil taxa supported the monophyly of the suborder Odontoceti including the sperm whale (Heyning 1997).

In this chapter the hypotheses of Milinkovitch et al. (1993, 1994), of a sister relationship between sperm whales and baleen whales, and more specifically and Gullberg (1994, 1996), of five primary evolutionary lineages of extant cetaceans, were tested with the use of 495-base pair sequences of the cytochrome c oxidase subunit I (COI) and combinations of this sequence with those of the cytochrome b (Cytb), 12S and 16S genes.

3.2. Material and Methods

3.2.1. Samples

Tissue (muscle, liver or heart) samples of 15 cetacean species were obtained from Brazil and Canada. All Canadian samples were provided by Dr. Jon Lien, of the Whale Research Group (WRG) at the Memorial University of Newfoundland (MUN), St. John's, Newfoundland (NF). Samples from Brazil (BR) were collected by myself and various other collectors at several locations in Brazil (Table 3.1). The sample of *Hippopotamus amphibius* was from the San Diego Zoo, San Diego, California.

Table 3.1. Number of samples, date and location of collection of samples of cetaceans species used in this study.

Species	# Samples	Date of collection	Location
Balaenoptera musculus	01	Apr 92	Crab River, Newfoundland
B. acutorostrata	01	27 Aug 89	Portugal Cove, NF
Megaptera novaeangliae	02	21 Jun 90	Newfoundland
		Jan 95	Rio de Janeiro, BR
Physeter macrocephalus	02	12 May 95	Prainha, BR
		01 Feb 96	Barra Nova, BR
Mesoplodon bidens	01	31 Aug 86	Newfoundland
Pontoporia blainvillei	02	31 Oct 94	Rio Grande, BR
		Mar 95	Rio de Janeiro, BR
Phocoena phocoena	01	Jan 93	Newfoundland
Delphinapterus leucas	02	03 May 89	Chance Cove, NF
		13 Jun 90	St. Anthony, NF
Delphinus delphis	01	28 Apr 89	Newfoundland
Lagenorhynchus acutus	01	10 Oct 94	Arnold's Cove, NF
L. albirostris	01	Jan 95	Chance Cove, NF
Peponocephala electra	01	24 May 95	Pecém, BR
Sotalia fluviatilis	02	04 Dec 94	Fortaleza, BR
		09 Jun 96	Taíba, BR
Stenella frontalis	01	26 Apr 96	Icaraí, BR
Tursiops truncatus	01	22 Nov 96	Fortaleza, BR

3.2.2. DNA extraction

DNA was extracted with the same procedure as in 2.2.2.

3.2.3. DNA amplification

PCR (polymerase chain reaction) was used to amplify 495-base pair sequences of the mitochondrial DNA cytochrome oxidase I (COI) gene from the collected samples. The primers used were COIf-L (5'-CCTGCAGGAGGAGGAGAYCC-3') and COIe-H (5'-CCAGAGATTAGAGGGAATCAGTG-3') (Kessing et al. 1989). Amplification reactions were performed according to 2.2.3.

3.2.4. Purification of PCR product

DNA was purified with the same procedure as in 2.2.4.

3.2.5. DNA Sequencing

The DNA sequencing procedure was identical to that described in 2.2.5.

3.2.6. Phylogenetic Analyses

Initially, the cytochrome oxidase I (COI) sequences were analyzed based on sequence data of 16 species from seven families of all the major taxonomic groups of extant cetaceans: superfamilies Platanistoidea, Delphinoidea, Ziphioidea, and Physeteroidea (sub-order Odontoceti), and family Balaenopteridae (sub-order Mysticeti). The sequence of the fin whale (*Balaenoptera physalus*) (GenBank accession number X61145) was from Arnason et al. (1991).

DNA sequences of seven non-cetacean mammals species were included as outgroups: these included four artiodactyls [hippopotamus (*Hippopotamus amphibius*) (495-bp COI gene sequenced by myself), cow (*Bos taurus*: GenBank accession number V00654)(Anderson et al. 1982), mule deer (*Odocoileus hemionus*) and caribou (*Rangifer tarantus*) (Carr 1998)]: two perissodactyls [the horse (*Equus caballus*) (Xu and Arnason 1994: X79547) and the greater Indian rhinoceros (*Rhinoceros unicornis*) (Xu et al. 1996: X97336)]: and a menotyphlan insectivore, the hedgehog (*Erinaceus europaeus*) (Krettek et al. 1995: X88898). The hedgehog was chosen as the external outgroup for the analyses because it is the species most distantly related to the cetaceans.

For subsequent analyses seven other data sets were used, including all the possible combinations of the COI gene together with the 12S and 16S rRNA, and cytochrome b (Cytb) genes. DNA sequences of the 12S, 16S, and Cytb genes were obtained from the National Center for Biotechnology Information (NCBI) GenBank data base (National Library of Medicine, Bethesda, MD).

The Cytb sequences of cetaceans used in the combined analyses were from Arnason and Gullberg (1994, 1996), and the 12S and 16S cetaceans sequences were from Milinkovitch et al. (1993, 1994). The Cytb sequences of *Odocoileus hemionus* and *H. amphibius* used here were from Irwin et al. (1991) and Montgelard et al. (1997), respectively. The 12S and 16S sequences of *Odocoileus* species were from Miyamoto et al. (1990). The Cytb, 12S and 16S sequences of *B. taurus* were from Anderson et al. (1982), *E. caballus* sequences were from Xu and Arnason (1994), *R.*

unicornius sequences were from Xu et al. (1996), and *E. europaues* sequences were from Krettek et al. (1995).

Phylogenetic analyses were performed with the Phylogenetic Analysis Using Parsimony (PAUP) [versions 4.0d59, 4.0d60, 4.0d61], program of Swofford (1997).

Maximum parsimony (MP) trees were obtained with the heuristic search algorithm, tree-bisection-and-reconnection option, with 10 random addition and delayed-character-transformation optimization. Ratios of transversions (Tv) to transitions (Ti) of 3:1, 10:1, and transversion only, were used for all data sets analyzed. Bootstrap analyses (Felsenstein 1985) were performed by means of the heuristic search algorithm with 10 random taxon additions and the tree-bisection-and-reconnection option in each of 300 replicates.

Neighbor-joining (NJ) analyses (Saitou and Nei 1987) were performed in all data sets using distance matrices calculated with Tamura-Nei, Kimura 2-parameters, and maximum-likelihood parameters models in PAUP (Swofford 1997). Bootstrapping for NJ trees were performed using the same parameters used for MP analyses, except for the number of replicates (1,000 replicates), and the gamma distribution shape parameter (γ), estimated after the maximum likelihood analysis.

Maximum-likelihood (ML) method (Felsenstein 1981) calculations, were performed on the COI data set and on all the combined data sets, using the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985) in PAUP (Swofford 1997). The ratio of Tv to Ti and the gamma shape parameter (γ) were first estimated by heuristic search and then used for the bootstrap analysis (300 replicates).

3.3. RESULTS

3.3.1. Phylogenetic analyses of COI sequences

Among the 495-bp of the COI sequences of the cetaceans (Figure 3.1) and seven non-cetacean species, 164 characters were parsimony-informative (Table 3.2). The number of trees, trees length, consistency index (CI), and retention index (RI) for the three different choices of character weighting are also shown in Table 3.2. One of the trees obtained by the MP analyses of all 23 taxa, with ratio of transitions to transversions of 3:1, is shown in Figure 3.2. The other two trees differ from that one only for the positions of the three Balaenopteridae species.

The results of the MP, NJ and ML analyses of the COI sequences support the hypothesis of a monophyletic group constituted by the order Cetacea. In all analyses, no sister relationship between the sperm whale and the baleen whales was identified.

The hippopotamus was observed to be the artiodactyl most closely related to the cetaceans according to MP analyses of COI gene sequences (Figure 3.2). This close relationship between hippopotamus and cetaceans was supported by bootstrap values of 55, 59 and 60, when transversions were weighted three and 10 times more than transitions, and when only transversions where considered respectively. However, NJ distance analyses did not show if either the *Hippopotamus* or the ruminant genera (*Bos*, *Odocoileus*, and *Rangifer*) are the closest relatives to cetaceans (Figure 3.3). ML analyses also did not resolve which artiodactyl group is more closely related to Cetacea (Figure 3.4).

Figure 3.1. DNA sequence variation in a 495-bp region of the cytochrome oxidase I mitochondrial gene of fifteen species of cetaceans and of *Hippopotamus amphibius*, obtained in this study. Dots represent nucleotides that are identical to the blue whale (*Balaenoptera musculus*) sequence. The top line gives the inferred amino acid sequence according to the single letter code of the International Union of Biochemists (IUB). Numbers at the end of the first and second line indicate the position numbers in the protein and nucleotide sequences, respectively. The cetacean species corresponding to the genera listed are: *Megaptera*: *M. novaeangliae*; *Physeter*: *P. macrocephalus*; *Mesoplodon*: *M. bidens*; *Pontoporia*: *P. blainvillei*; *Delphinapterus*: *D. leucas*; *Phocoena*: *P. phocoena*; *Peponocephala*: *P. electra*; *Delphinus*: *D. delphis*; *Tursiops*: *T. truncatus*; *Stenella*: *S. frontalis*; and *Sotalia*: *S. fluviatilis*.

	I	L	Y	Q	H	L	F	W	F	F	10
B.musculus	atc	ctg	tat	caa	cac	tta	ttc	tga	ttc	ttt	30
B.acutorostrara	...	...	..c	...	...	c..	...	...	...	...	
Megaptera	...	..a	...	...	...	c..	...	...	..t	...	
Physeter	...	...	...	...	...	...	...	...	...	..c	
Mesoplodon	...	...	...	...	...	c.g	...	...	...	...	
Pontoporia	...	a.c	...	...	...	c.g	...	...	..t	...	
Delphinapterus	...	..a	...	...	...	c..	...	...	..t	...	
Phocoena	...	..a	...	...	...	...	...	...	..t	...	
Peponocephala	...	t.a	...	...	...	c.g	...	...	..t	...	
L.acutus	...	t.a	..c	...	...	..g	...	...	..t	...	
L.albirostris	...	t.a	...	...	...	..g	...	...	..t	...	
Delphinus	...	..a	...	...	...	c..	...	...	..t	...	
Tursiops	...	t.a	...	...	...	...	...	...	..t	...	
Stenella	...	t.a	..c	...	...	...	...	...	..t	...	
Sotalia	...	t.a	..c	...	...	...	...	...	..t	...	
Hippopotamus	..t	..a	...	...	...	c..	...	...	...	..c	

	G	H	P	E	V	Y	I	L	I	L	20
B.musculus	ggt	cac	cct	gaa	gta	tac	att	cta	att	ctc	60
B.acutorostrara	...	...	..c	...	...	..t	..c	...	...	...	
Megaptera	...	...	..c	...	...	..t	...	...	...	..a	
Physeter	...	...	...	..g	..c	..t	...	...	..c	..a	
Mesoplodon	...	..t	..c	...	...	...	...	..g	..c	..a	
Pontoporia	...	..t	..c	...	..g	..t	..c	..g	...	..a	
Delphinapterus	...	...	..c	...	...	..t	..c	...	...	..a	
Phocoena	...	...	...	...	..g	..t	...	...	...	..a	
Peponocephala	...	..t	..c	...	...	..t	..c	t..	...	..a	
L.acutus	...	..t	..a	...	...	..t	...	t..	...	..a	
L.albirostris	...	..t	...	...	...	..t	...	t..	...	..a	
Delphinus	...	...	..c	...	...	..t	..c	...	...	..a	
Tursiops	...	..t	...	...	...	..t	...	t..	...	..a	
Stenella	..c	..t	...	...	...	..t	...	t..	...	..a	
Sotalia	...	..t	..c	...	...	...	...	t..	...	..a	
Hippopotamus	..a	...	..c	...	...	...	..c	..g	..c	..g	

	P	G	F	G	M	I	S	H	I	V	30
B.musculus	cct	ggg	ttc	gga	ata	att	tca	cac	att	gtg	90
B.acutorostrara	...	...	...	...	...	...	...	...	...	...	
Megaptera	..c	...	...	..g	...	..c	...	..t	..c	..a	
Physeter	...	..c	...	...	...	..c	..c	...	..c	..a	
Mesoplodon	..c	..t	..t	..g	...	..c	...	..t	..c	..a	
Pontoporia	..c	...	..t	...	..g	...	...	..t	..c	..a	
Delphinapterus	..c	..c	...	..g	...	..c	...	..t	..c	..a	
Phocoena	..c	..t	...	...	...	..c	..g	...	..c	..a	
Peponocephala	..c	...	..t	...	...	...	...	..t	..c	..t	
L.acutus	..a	..t	...	...	...	...	...	...	..c	.at	
L.albirostris	..c	..t	..t	...	...	...	...	...	..c	..t	
Delphinus	..c	..c	...	..g	...	..c	...	..t	..c	..a	
Tursiops	...	..c	..t	...	...	...	...	...	..c	..t	
Stenella	..c	..c	..t	...	...	...	...	...	..c	..t	
Sotalia	..c	..c	..t	...	...	...	...	..t	..c	..t	
Hippopotamus	..c	..t	...	...	...	..c	..g	...	...	..a	

	T	Y	Y	S	G	K	K	E	P	F	40
B.musculus	act	tat	tac	tca	gga	aaa	aaa	gaa	cct	ttc	120
B.acutorostrara	...	...	...	...	...	...	...	...	...	...	
Megaptera	..c	...	...	..g	...	...	...	..g	...	..t	
Physeter	...	...	..t	..c	..g	...	...	..g	..c	..t	
Mesoplodon	..c	...	...	...	...	...	...	...	...	...	
Pontoporia	...	..c	...	...	...	...	...	...	...	...	
Delphinapterus	..c	..c	...	..g	...	...	...	..g	..c	..t	
Phocoena	..a	..c	..t	...	...	...	...	..g	...	..t	
Peponocephala	...	...	..t	...	...	...	...	...	...	...	
L.acutus	..c	...	..t	..g	..g	...	...	..g	...	..t	
L.albirostris	...	...	..t	...	..g	...	...	...	...	..t	
Delphinus	..c	..c	...	..g	...	...	...	..g	..c	..t	
Tursiops	...	...	..t	...	..g	...	...	...	...	..t	
Stenella	...	...	..t	...	..g	...	...	...	...	..t	
Sotalia	...	...	..t	...	..g	...	...	...	..c	..t	
Hippopotamus	..a	..c	...	..c	...	...	...	...	...	...	

	G	Y	M	G	M	V	W	A	M	V	50
B.musculus	ggc	tat	atg	gga	atg	gtc	tga	gct	atg	gtg	150
B.acutorostrara	..t	...	..a	...	..a	...	...	...	..a	..a	
Megaptera	..a	...	..a	..g	...	..g	...	...	..a	a.a	
Physeter	..a	...	...	...	..a	...	..g	...	..a	a.c	
Mesoplodon	..g	..c	..a	...	..a	..t	..g	..c	..a	..c	
Pontoporia	..a	...	...	..g	..a	...	...	...	..a	a.c	
Delphinapterus	..a	...	..a	..g	...	..g	..g	...	..a	a.t	
Phocoena	..g	...	..a	..g	...	..a	...	...	...	..c	
Peponocephala	..a	...	..a	..g	..a	..a	...	...	..a	...	
L.acutus	..g	..c	..a	...	..a	..a	..g	..c	..a	..t	
L.albirostris	..g	...	...	...	..a	..a	...	...	..a	..t	
Delphinus	..a	...	..a	..g	...	..g	..g	...	..a	a.t	
Tursiops	..g	...	...	...	..a	..a	...	...	..a	..t	
Stenella	..g	...	...	...	..a	..a	...	...	..a	..t	
Sotalia	..g	...	...	...	..a	..a	..g	...	...	..t	
Hippopotamus	..g	..c	..a	..c	..a	...	...	...	..a	a.a	

	S	I	G	F	L	G	F	I	V	W	60
B.musculus	tcc	atc	gga	ttc	tta	ggc	ttt	atc	gta	tga	180
B.acutorostrara	...	..t	..g	..t	...	...	...	...	...	...	
Megaptera	..t	..t	..g	..t	c..	...	..c	...	...	...	
Physeter	..t	..t	...	...	...	...	...	...	...	...	
Mesoplodon	..t	...	...	...	c..	...	...	...	...	...	
Pontoporia	..t	...	...	...	c..	..t	...	...	...	...	
Delphinapterus	..t	..t	..c	...	c..	..t	..c	..t	...	...	
Phocoena	..t	...	..t	...	c..	..t	...	...	...	...	
Peponocephala	..t	..t	..c	...	c..	..t	..c	..t	...	...	
L.acutus	..t	..t	..t	...	c..	..t	..c	..t	...	...	
L.albirostris	..t	..t	..t	...	c..	..t	..c	..t	...	...	
Delphinus	..t	..t	..c	...	c..	..t	..c	..t	...	...	
Tursiops	..t	..t	..t	...	c..	..t	...	..t	...	...	
Stenella	..t	..t	..t	...	c..	..t	..c	..t	...	...	
Sotalia	..t	...	..t	...	c..	..t	..c	..t	...	...	
Hippopotamus	..t	...	..g	...	c..	..a	...	..t	...	...	

	A	H	H	M	F	T	V	G	M	D	70
B.musculus	gcc	cac	cat	atg	ttt	aca	gta	ggt	ata	gac	210
B.acutorostrara	...	..t	..c	...	...	...	...	...	...	...	
Megaptera	..t	..t	...	..a	...	...	..c	..a	...	...	
Physeter	...	...	..c	..a	..c	..t	...	...	..g	..t	
Mesoplodon	...	...	...	...	...	...	..c	..a	..g	...	
Pontoporia	...	...	...	..a	..c	...	..c	..a	...	...	
Delphinapterus	..t	...	...	..a	...	...	..c	..a	...	...	
Phocoena	...	..t	..c	..a	...	...	..t	..a	...	...	
Peponocephala	..t	..t	..c	...	...	...	..t	..a	...	...	
L.acutus	..t	...	..c	...	...	...	..t	..a	...	...	
L.albirostris	..t	..t	...	..a	..c	...	..t	..a	...	...	
Delphinus	..t	...	...	..a	...	...	..c	..a	...	...	
Tursiops	...	..t	...	...	..c	...	..t	..a	...	...	
Stenella	..t	..t	...	...	..c	...	..t	..a	...	...	
Sotalia	...	..t	...	...	...	...	..t	..a	...	..t	
Hippopotamus	...	..t	..c	..a	...	...	...	...	...	...	

	V	D	T	R	A	Y	F	T	S	A	80
B.musculus	gtt	gat	aca	cga	gca	tat	ttc	aca	tcg	gct	240
B.acutorostrara	...	..c	...	...	...	...	...	...	..a	...	
Megaptera	...	..c	...	...	...	...	...	...	..a	...	
Physeter	...	..c	...	...	...	..c	..t	...	..c	..a	
Mesoplodon	..c	..c	...	...	...	...	..t	...	..a	...	
Pontoporia	..a	..c	...	...	...	..c	...	...	..a	..c	
Delphinapterus	..a	..c	...	...	...	...	...	...	..a	...	
Phocoena	..a	...	...	...	...	..c	..t	...	..a	..c	
Peponocephala	..a	..c	...	...	...	...	..t	...	..a	...	
L.acutus	..a	..c	...	...	...	...	..t	...	..t	...	
L.albirostris	..g	..c	...	...	...	...	..t	...	..a	...	
Delphinus	..a	..c	...	...	...	...	...	...	..a	...	
Tursiops	..g	...	...	...	...	...	..t	...	..a	...	
Stenella	..g	..c	...	...	...	...	...	...	..a	...	
Sotalia	..a	..c	...	...	...	...	..t	...	..a	...	
Hippopotamus	..c	..c	..c	...	...	..c	...	...	..c	..c	

	T	M	I	I	A	I	P	T	G	V	90
B.musculus	act	ata	att	att	gct	att	ccc	aca	gga	gta	270
B.acutorostrara	...	...	..c	...	..c	...	...	...	...	...	
Megaptera	..c	...	...	...	...	...	...	...	...	...	
Physeter	...	...	..c	...	..c	..c	..t	...	...	...	
Mesoplodon	..c	...	..c	...	...	..c	..t	...	...	..c	
Pontoporia	..c	...	...	...	..c	...	...	...	...	...	
Delphinapterus	..c	...	...	...	...	...	...	...	..g	...	
Phocoena	...	...	..c	...	...	..c	..t	...	...	...	
Peponocephala	...	...	..c	..c	..a	...	...	...	...	...	
L.acutus	...	...	...	..c	..a	...	..t	...	...	...	
L.albirostris	...	...	...	..c	..a	...	..t	...	...	...	
Delphinus	..c	...	...	...	...	...	...	...	..g	...	
Tursiops	...	...	...	..c	..a	...	..t	...	...	...	
Stenella	...	...	...	..c	..a	...	..t	...	...	...	
Sotalia	...	...	...	..c	..a	...	..t	...	...	...	
Hippopotamus	..c	...	...	..c	..c	..c	...	...	...	...	

	K	V	F	S	W	L	A	T	L	H	100
B.musculus	aaa	gtc	ttc	agt	tga	tta	gca	aca	cta	cac	300
B.acutorostrara	...	...	...	..c	...	...	...	...	...	...	
Megaptera	...	...	...	...	...	...	...	...	...	...	
Physeter	...	a..	...	..c	...	c.g	...	..c	..c	..t	
Mesoplodon	...	..t	...	..c	...	c..	...	..c	..t	..t	
Pontoporia	...	..a	...	...	...	c..	...	...	..t	...	
Delphinapterus	...	...	..t	..c	...	c.g	...	...	..c	...	
Phocoena	...	...	..t	..c	...	c.g	...	...	..t	..t	
Peponocephala	...	..t	...	...	...	c.g	...	...	..t	...	
L.acutus	...	..t	...	..c	...	c..	...	...	..t	...	
L.albirostris	...	..t	...	...	...	c..	...	...	..t	...	
Delphinus	...	...	..t	..c	...	c.g	...	...	..c	...	
Tursiops	...	..t	...	...	...	c..	..g	...	..t	...	
Stenella	...	..t	...	...	...	c..	...	...	..t	...	
Sotalia	...	..t	...	...	...	c.g	...	...	..t	..t	
Hippopotamus	...	..a	...	..c	...	c..	...	...	..g	..t	

	G	G	N	I	K	W	S	P	A	L	110
B.musculus	gga	ggt	aat	att	aaa	tga	tct	cct	gct	cta	330
B.acutorostrara	...	...	..c	...	...	...	...	...	...	...	
Megaptera	...	...	...	...	...	...	...	...	...	...	
Physeter	...	..c	..c	...	...	...	...	..c	..c	...	
Mesoplodon	...	..a	..c	..c	...	...	...	..c	...	t..	
Pontoporia	...	...	...	...	...	...	...	..c	..c	t..	
Delphinapterus	...	..g	...	...	...	...	...	..a	..c	...	
Phocoena	...	..a	...	..c	...	...	...	..c	..c	...	
Peponocephala	...	..a	...	...	...	...	...	..c	..c	...	
L.acutus	...	..a	...	..c	...	...	...	..c	..c	t..	
L.albirostris	...	..a	...	...	...	...	...	...	..c	...	
Delphinus	...	..g	...	...	...	...	...	..a	..c	...	
Tursiops	...	..a	...	...	...	...	...	...	..c	...	
Stenella	...	..a	...	...	...	...	...	...	..c	...	
Sotalia	...	..a	...	...	...	...	...	...	..c	...	
Hippopotamus	...	..g	..c	..c	...	..g	..c	...	...	a.g	

	M	W	A	L	G	F	I	F	L	F	120
B.musculus	atg	tgg	gcc	ctg	ggt	ttc	atc	ttc	ctt	ttc	360
B.acutorostrara	..a	..a	...	..a	...	...	...	...	..c	...	
Megaptera	...	...	...	..a	...	...	...	...	...	...	
Physeter	..a	..a	...	t.a	..c	...	..t	..t	..c	..t	
Mesoplodon	..a	..a	...	t.a	..c	..t	..t	...	..c	..t	
Pontoporia	..a	..a	...	..a	..c	..t	...	...	..g	..t	
Delphinapterus	..a	..a	...	..a	..c	..t	..t	...	..a	...	
Phocoena	..a	...	...	...	..c	..t	..t	...	..a	...	
Peponocephala	..a	..a	...	..a	..c	..t	...	...	t.a	...	
L.acutus	..a	..a	..a	..a	..c	..t	..t	...	t.a	..t	
L.albirostris	..a	..a	..t	..a	..c	..t	...	...	t.a	...	
Delphinus	..a	..a	...	..a	..c	..t	..t	...	..a	...	
Tursiops	..a	..a	..t	..a	..c	..t	...	...	t.g	...	
Stenella	..a	..a	..t	..a	..c	..t	...	...	t.a	...	
Sotalia	..a	..a	...	..a	..c	..t	..t	...	t.a	...	
Hippopotamus	...	..a	...	..a	..c	..t	..t	...	..a	...	

```
                T   V   G   G   L   T   G   I   V   L   130
B.musculus      aca gta ggc ggc cta acc ggt atc gtt cta   390
B.acutorostrara ... ... ... ... ... ..t ... ..t ..c ...
Megaptera       ... ... ... ... ... ... ... ... ... ...
Physeter        ... ... ..t ... t.g ..t ... ..t ..c ...
Mesoplodon      ..t ... ..t ... ... ... ..c ..t ..c ..g
Pontoporia      ... ... ..g ... t.. ..t ... ..t a.. ...
Delphinapterus  ... ... ..a ... t.. ... ... ..t a.c ...
Phocoena        ... ... ..t ..t t.. ..t ... ..t a.c ..g
Peponocephala   ... ... ..t ..t t.. ... ... ..t a.c ...
L.acutus        ..g ... ..a ..t t.. ... ... ..t a.c ...
L.albirostris   ... ... ..a ..t t.. ... ... ..t a.c ...
Delphinus       ... ... ..a ... t.. ... ... ..t a.c ...
Tursiops        ... ... ..a ..t t.. ... ... ... a.c ...
Stenella        ... ... ..a ..t ... ... ... ... a.c ...
Sotalia         ... ... ..t ..t t.. ... ... ..t a.c ...
Hippopotamus    ... ... ..t ... ... ..a ... ..t ... ..c

                A   N   S   S   L   D   I   V   L   H   140
B.musculus      gcc aac tca tca cta gat att gtc cta cac   420
B.acutorostrara ... ... ... ... ... ... ... ... ... ...
Megaptera       ... ... ... ... ... ... ... ... ... ...
Physeter        ... ... ..t ... ... ... ..c ... ..t ...
Mesoplodon      ... ... ... ..c t.. ..c ... ..a ..c ..t
Pontoporia      ... ... ... ..c t.. ... ... ... ..t ..t
Delphinapterus  ... ... ... ..c t.. ..c g.c a.. ..c ...
Phocoena        ..t ... ... ..c ... ... g.. a.. ..t ...
Peponocephala   ..t ... ..g ..c ... ... ..c a.t ..c ...
L.acutus        ..t ... ... ..c ... ... ..c a.t ..c ...
L.albirostris   ..t ... ... ..c t.. ... ... a.. ..t ..t
Delphinus       ... ... ... ..c t.. ..c g.c a.. ..c ...
Tursiops        ..t ..t ... ..c ... ... ... a.. ..t ..t
Stenella        ..t ... ... ..c ... ... ..c a.. ..t ..t
Sotalia         ..t ... ... ..t ... ... ..c a.. ..c ...
Hippopotamus    ... ... ... ..c ... ... ... ..t ..c ...

                D   T   Y   Y   V   V   A   H   F   H   150
B.musculus      gat act tac tac gta gtt gcc cac ttc cac   450
B.acutorostrara ..c ..c ... ... ... ... ..t ..t ... ...
Megaptera       ... ... ... ... ... ... ... ... ... ...
Physeter        ... ..a ... ..t ... ..c ... ... ... ...
Mesoplodon      ... ... ..t ... ... ... ... ..t ... ...
Pontoporia      ..c ..c ..t ..t ... ... ... ... ... ...
Delphinapterus  ..c ..a ..t ..t ... ..c ..a ... ... ...
Phocoena        ... ..c ... ... ... ... ... ..t ... ...
Peponocephala   ..c ..c ..t ..t ... ... ..t ..t ..t ...
L.acutus        ..c ..c ..t ..t ... ... ... ..t ..t ...
L.albirostris   ..c ..c ..t ..t ... ... ..t ..t ... ...
Delphinus       ..c ..a ..t ..t ... ..c ..a ... ... ...
Tursiops        ..c ..c ... ..t ..g ... ..t ..t ..t ...
Stenella        ..c ..c ..t ..t ... ... ..t ..t ..t ...
Sotalia         ..c ..c ..t ..t ..g ... ... ..t ..t ...
Hippopotamus    ... ..c ..t ... ... ... ..a ..t ... ...
```

	Y	V	L	S	M	G	A	V	F	A	160
B.musculus	tat	gtt	cta	tca	ata	gga	gca	gtc	ttc	gcc	480
B.acutorostrara	...	..a	t..	...	...	...	...	...	...	...	
Megaptera	...	...	...	...	...	...	...	...	...	...	
Physeter	..c	...	..t	...	...	...	..t	..t	..t	...	
Mesoplodon	...	..a	..c	...	...	...	..t	...	..t	...	
Pontoporia	...	..g	..t	...	...	...	..t	...	...	...	
Delphinapterus	...	..g	...	...	...	..g	..t	...	...	...	
Phocoena	...	..c	..t	...	..g	..g	..t	..t	...	...	
Peponocephala	...	..a	..t	...	...	...	..t	...	..t	...	
L.acutus	...	..g	..c	...	...	...	..t	...	..t	...	
L.albirostris	...	..g	..t	...	...	...	..t	...	..t	...	
Delphinus	...	..g	...	...	...	..g	..t	...	...	...	
Tursiops	...	..g	..t	...	...	...	..t	...	..t	...	
Stenella	..c	..g	..t	...	...	...	..t	...	..t	...	
Sotalia	...	..a	..t	...	...	...	..t	...	..t	...	
Hippopotamus	...	...	...	...	...	..g	..t	...	..t	...	

	I	M	G	G	F		165
B.musculus	att	ata	gga	ggc	ttt		495
B.acutorostrara	..c	..g	...	..t	...		
Megaptera	...	...	...	...	...		
Physeter	..c	...	...	..t	..c		
Mesoplodon	..c	...	...	..a	...		
Pontoporia	...	...	...	...	...		
Delphinapterus	..c	...	...	..t	..c		
Phocoena	...	...	...	...	...		
Peponocephala	..c	...	...	..t	...		
L.acutus	..c	...	...	...	..c		
L.albirostris	..c	...	...	...	..c		
Delphinus	..c	...	...	..t	..c		
Tursiops	..c	...	...	...	..c		
Stenella	..c	...	...	...	..c		
Sotalia	..c	...	...	..t	..c		
Hippopotamus	...	...	..g	..g	...		

All phylogenetic analyses of the COI sequences identified four clades corresponding to four major groups within the order Cetacea, identified as: Physeteridae, Ziphiidae, infraorder Delphinida, and suborder Mysticeti. The infraorder Delphinida was represented by the three families of the superfamily Delphinoidea (Delphinidae, Phocoenidae, Monodontidae) and the family Pontoporidae from the superfamily Platanistidae. The order Mysticeti was represented by species from the family Balaenopteridae.

The results show that the family Delphinidae and the superfamily Delphinoidea were both monophyletic, however, phylogenetic relationships within Delphinoidea were not resolved. A close relationship between Pontoporidae and Delphinoidea was also supported, but the bootstrap values were low. A monophyletic Mysticeti clade was strongly supported in the MP analyses by bootstrap values of 94, 89, and 84, for transversion:transition (Tv:Ti) ratios of 3:1, 10:1, and transversions only, as well as by the relationships among its members, represented by four Balaenopteridae species (Figure 3.2).

A close relationship between baleen whales (Balaenopteridae) and the sperm whales (*Physeter*) was not identified in any of the MP analyses. No sister relationship was also identified between the beaked whales (*Mesoplodon*) and the baleen whales, nor between the beaked whales and the sperm whales. The phylogenetic relationships among the four major cetacean lineages (Delphinida, Ziphinoidea, Physeteroidea, and Mysticeti) were not resolved on the basis of the MP analyses.

All three NJ bootstrap trees obtained were congruent with the maximum parsimony tree in Figure 3.2, except for the position of the Artiodactyla species. No support was observed for a Hippopotamus/Cetacea clade. The NJ bootstrap tree of maximum-likelihood distances is shown in Figure 3.3.

The ML analysis produced a single tree shown in Figure 3.4. Bootstrap values obtained after the estimation of the Tv:Ti ratio (6.12:1) and the γ shape parameter (γ=0.125) were lower than the bootstrap values yielded by the MP analyses. This tree was essentially similar to the tree in Figure 3.2, but it did not resolve which group of Artiodactyla was most closely related to Cetacea. The most parsimonious relationship of the four cetacean clades obtained by ML was (Mysticeti (Physeteridae, Ziphiidae (Delphinida))). However, that relationship was not supported by bootstrap analyses (Figure 3.4). A close relationship between sperm and beaked whales suggested by the MP analysis did not obtain bootstrap support, with bootstrap value of only 43%.

Figure 3.2. One of the three maximum parsimony tree (heuristic search, 300 replicates) based on the 495-bp sequences of the cytochrome oxidase I mitochondrial gene of 16 cetaceans, four artiodactyls, and two perissodactyls, with the hedgehog (order Lipotyphla) as outgroup. The other two trees differ from this one only in the positions of the three species of the genus *Balaenoptera*. The top numbers correspond to nucleotide differences between branches. The bottom values correspond to bootstrap values obtained by MP analysis in which transversions were weighted three times more than transitions. The species corresponding to the genera in this figure are the same indicated in Figure 3.1.

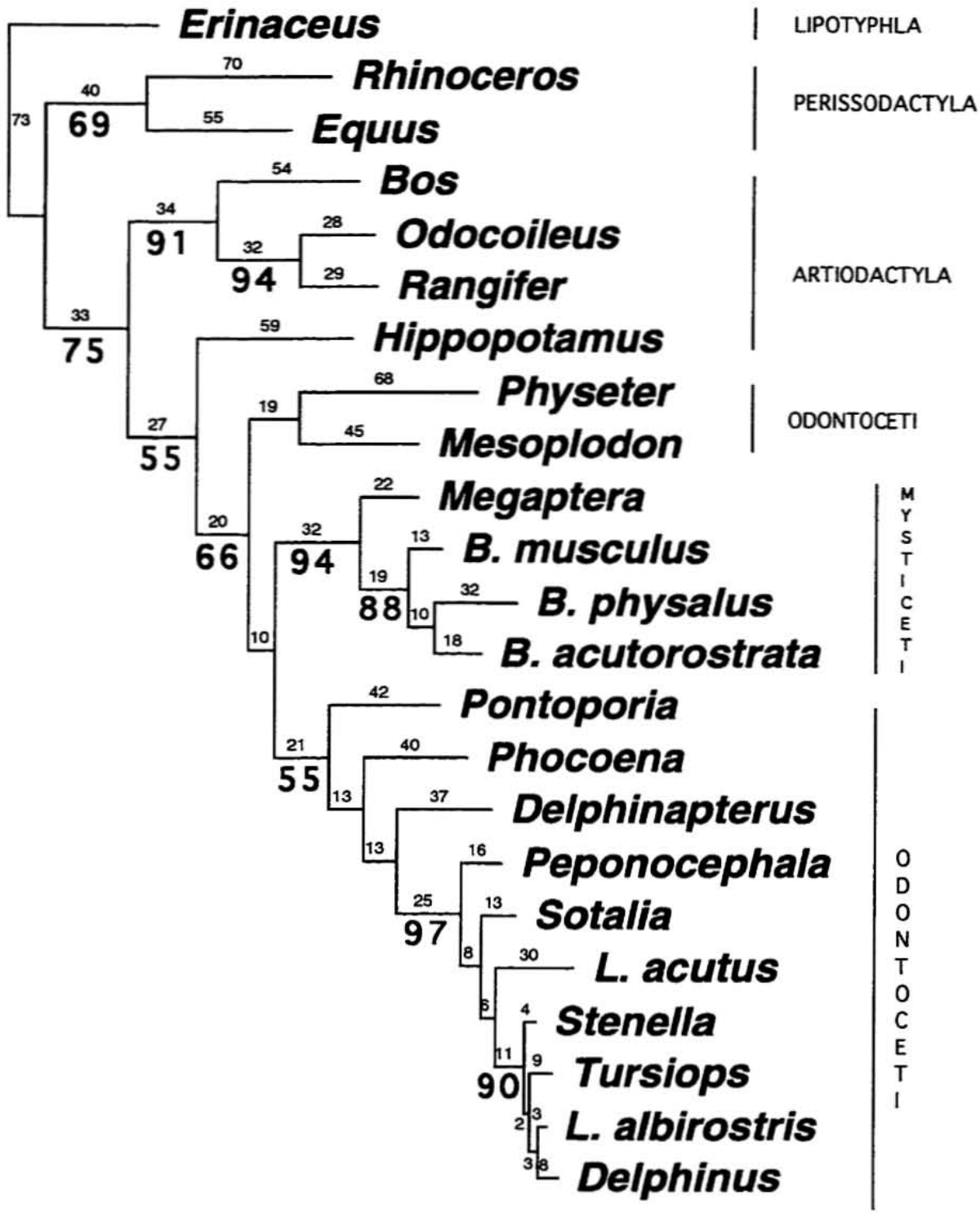

Erinaceus
Rhinoceros
Equus
Bos
Odocoileus
Rangifer
Hippopotamus
Physeter
Mesoplodon
Megaptera
B. musculus
B. physalus
B. acutorostrata
Pontoporia
Phocoena
Delphinapterus
Peponocephala
Sotalia
L. acutus
Stenella
Tursiops
L. albirostris
Delphinus
LIPOTYPHLA
PERISSODACTYLA
ARTIODACTYLA
ODONTOCETI
MYSTICETI
ODONTOCETI

Figure 3.3. Neighbor-joining tree of maximum-likelihood distances (Tv:Ti = 6.12:1. γ = 0.125) based on the 495-bp sequences of the cytochrome oxidase I mitochondrial gene of 16 cetaceans. and seven non-cetaceans used as outgroups. Numbers indicate bootstrap values (1.000 replicates). The species corresponding to the genera in this figure are the same indicated in Figure 3.1.

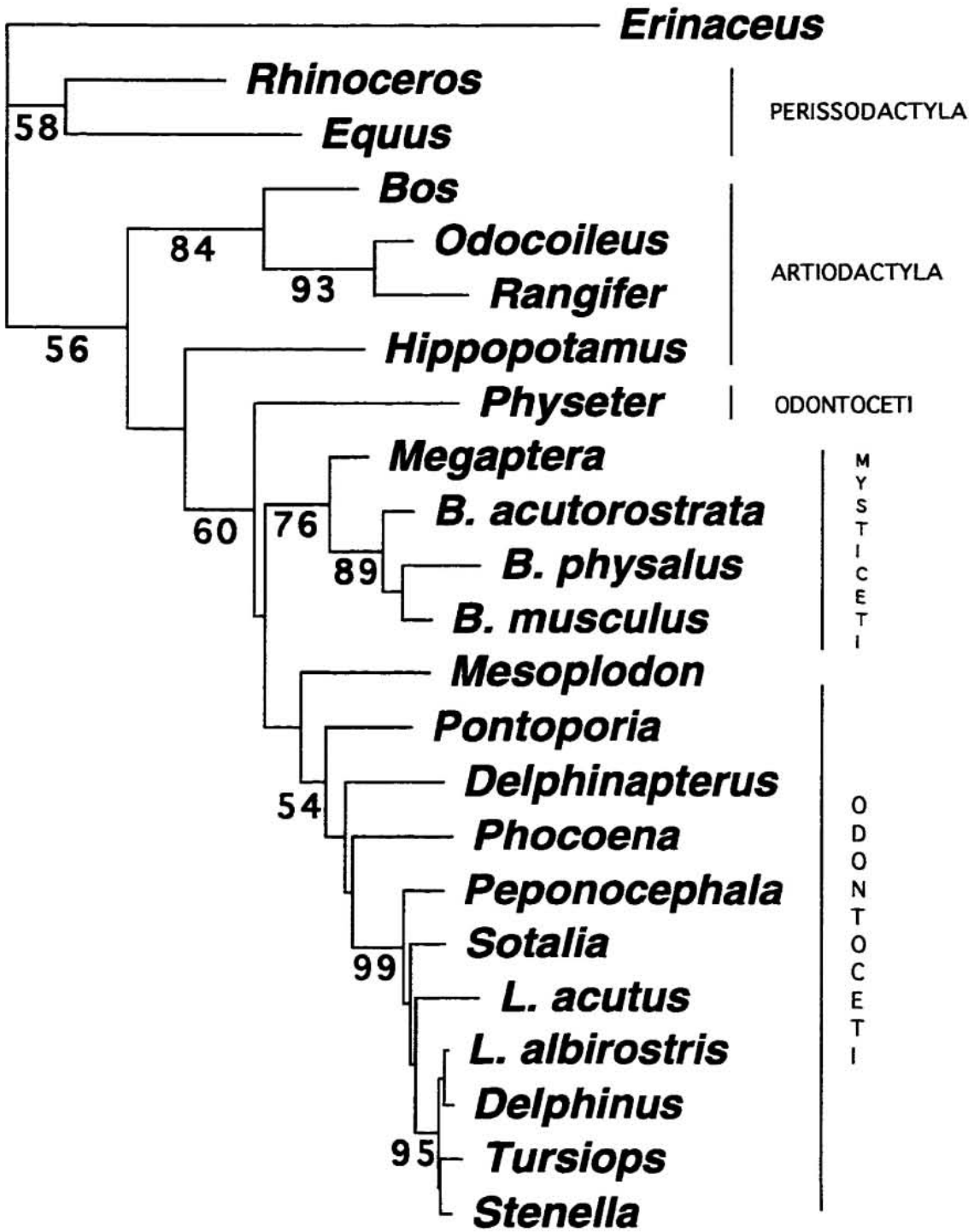

Erinaceus
Rhinoceros
Equus
58
PERISSODACTYLA
Bos
Odocoileus
Rangifer
84
93
ARTIODACTYLA
56
Hippopotamus
Physeter
ODONTOCETI
Megaptera
B. acutorostrata
B. physalus
B. musculus
60
76
89
MYSTICETI
Mesoplodon
Pontoporia
Delphinapterus
Phocoena
Peponocephala
Sotalia
L. acutus
L. albirostris
Delphinus
Tursiops
Stenella
54
99
95
ODONTOCETI

Figure 3.4. Maximum-likelihood tree based on the 495-bp sequences of the cytochrome oxidase I mitochondrial gene of 16 cetaceans, and seven non-cetaceans used as outgroups. Numbers indicate bootstrap values (300 replicates). The transversions were weighted 6.27:1 more than transitions (ratio estimated previously by heuristic search and likelihood options). The gamma shape distribution parameter was γ=0.125, which was also estimated by heuristic search. The species corresponding to the genera in this figure are the same indicated in Figure 3.1.

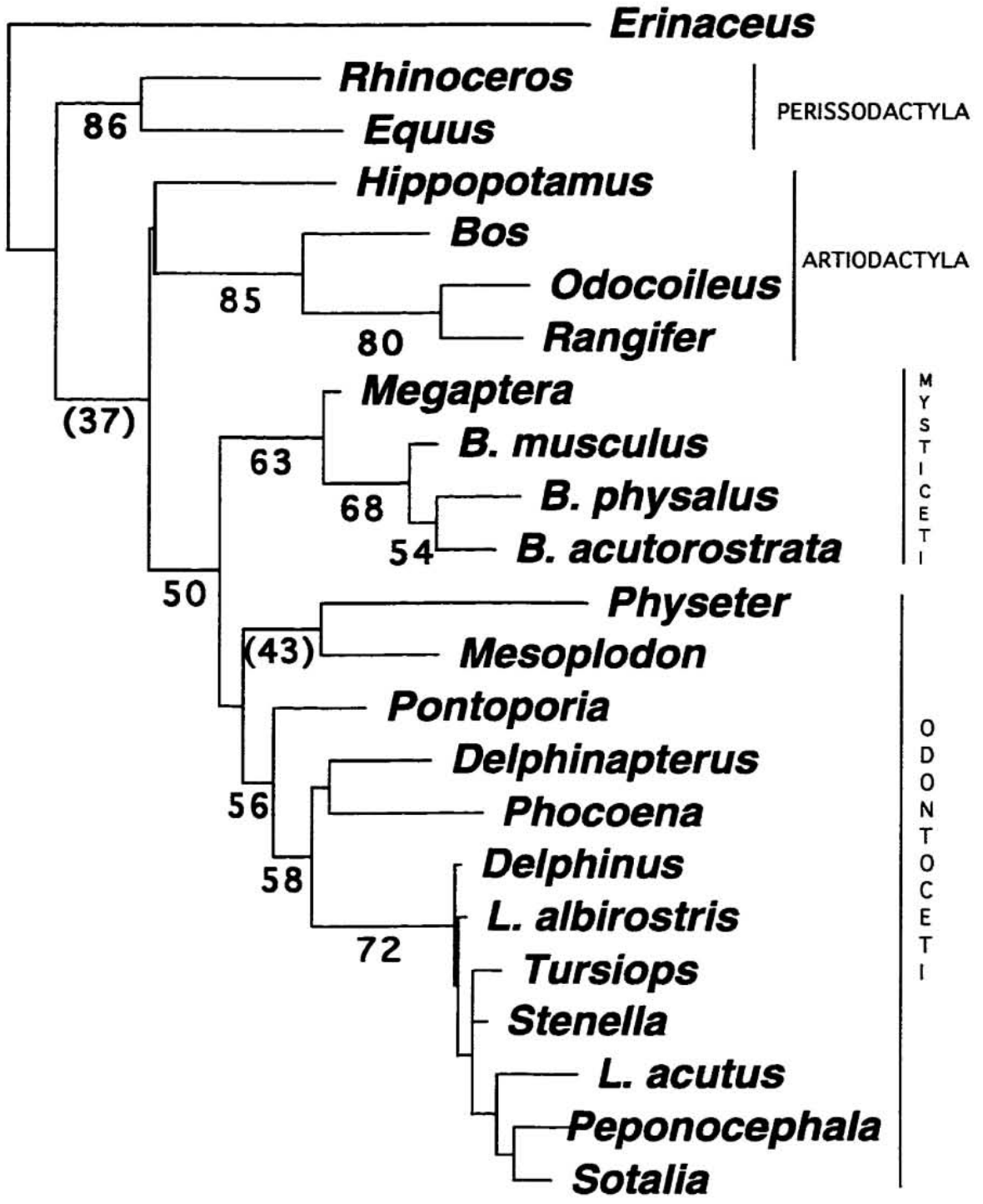

Erinaceus
Rhinoceros
Equus
86
PERISSODACTYLA
Hippopotamus
Bos
Odocoileus
Rangifer
85
80
ARTIODACTYLA
Megaptera
B. musculus
B. physalus
B. acutorostrata
(37)
63
68
54
MYSTICETI
50
Physeter
Mesoplodon
Pontoporia
Delphinapterus
Phocoena
Delphinus
L. albirostris
Tursiops
Stenella
L. acutus
Peponocephala
Sotalia
(43)
56
58
72
ODONTOCETI

Table 3.2. Parameters obtained by maximum parsimony (MP) and maximum likelihood (ML) analyses for the COI gene and all the gene combinations. Abbreviations: parsimony-informative characters (inf.char.); tree (tr); tree length (trl); consistency index (CI); retention index (RI), transitions (Ti); transversions (Tv); gamma distribution shape parameter (γ)

	#base	#inf.	MP 3:1				MP 10:1				MP Tv				M L	
Genes	pairs	char	#tr	trl	CI	RI	#tr	trl	CI	RI	#tr	trl	CI	RI	Tv:Ti	γ
COI	495	164	3	1146	0.38	0.53	1	2355	0.42	0.59	68	172	0.46	0.64	6.12:1	0.125
COI+12S	885	267	1	1650	0.51	0.47	1	3708	0.53	0.52	2	294	0.56	0.57	5.08:1	0.175
COI+16S	1018	264	1	1558	0.55	0.49	1	3525	0.58	0.55	1	281	0.61	0.60	4.63:1	0.151
COI+Cytb	1635	601	1	4103	0.46	0.46	1	9351	0.48	0.53	5	749	0.50	0.57	4.19:1	0.226
COI+12S+16S	1408	369	1	2133	0.58	0.52	1	4912	0.61	0.58	1	397	0.63	0.62	4.18:1	0.178
COI+12S+Cytb	2025	697	1	4415	0.51	0.48	1	10223	0.53	0.54	1	829	0.54	0.58	4.28:1	0.205
COI+16S+Cytb	2158	681	1	4094	0.54	0.49	1	9484	0.56	0.55	1	770	0.58	0.60	3.83:1	0.209
COI+12S+16S+Cyb	2548	786	1	4671	0.56	0.50	1	10880	0.58	0.56	1	887	0.59	0.60	3.82:1	0.195

Table 3.3. Summary of bootstrap results for maximum parsimony (MP), neighbor-joining (NJ), and maximum-likelihood (ML) analyses of 16 species of cetaceans and seven non-cetacean outgroups. A (+) indicates that the result supports the hypothesis that the sperm whale (*Physeter*) constitutes a monophyletic group together with the Mysticeti (Milinkovitch et al. 1993, 1994). Bootstrap support values are shown in parentheses. A (–) indicates such a hypothesis is not supported.

	#base	#inf.	MP			NJ			ML
Genes	pairs	char	3:1	10:1	Tv	TN	K2p	ML	HKY
COI	495	164	–	–	–	–	–	–	–
COI+12S	885	267	–	–	–	–	–	–	–
COI+16S	1018	264	–	–	–	–	–	–	–
COI+Cytb	1635	601	–	–	–	–	–	–	–
COI+12S+16S	1408	369	–	–	–	–	–	+ (70)	+ (64)
COI+12S+Cytb	2025	697	–	–	–	+ (55)	+ (52)	+ (62)	+ (60)
COI+16S+Cytb	2158	681	+ (52)	–	–	–	+ (64)	+ (82)	+ (56)
COI+12S+16S+Cytb	2548	786	+ (56)	–	–	+ (61)	+ (72)	+ (89)	+ (81)

3.3.2. Phylogenetic analyses of the combined gene sequences

All possible combinations of the COI gene sequence with the 12S, 16S, and the Cytb genes sequences (COI+12S, COI+16S, COI+Cytb, COI+12S+16S, COI+12S+Cytb, COI+16S+Cytb, and COI+12S+16S+Cytb) were analyzed using the same three methods (MP, NJ, and ML) used for the COI gene alone. In order to avoid misleading results due to different ratios of transversions to transitions in different genes (Hasegawa et al. 1991), MP analyses were performed in Tv:Ti ratios of 3:1, 10:1, and transversions only. For the ML analyses, the Tv:Ti ratio was estimated before bootstrapping, by heuristic search with no swapping, and using the HKY model (Hasegawa et al. 1985) in PAUP (Swofford 1997).

Table 3.2 shows the number of base pairs and parsimony-informative characters in each of the combined data sets. The longest sequence data set was represented by the combined COI+12S+16S+Cytb gene sequences, which contained 2,548 total base pairs and 786 parsimony-informative characters. The MP parameters (number of trees, tree lengths, CI, and RI) obtained for each of the Tv:Ti ratios analyzed, and the ML parameters estimated (Tv:Ti, and γ shape) are also shown in Table 3.2.

In only two of 21 MP analyses on the combined data sets (Table 3.3), was Milinkovitch's hypothesis (Milinkovitch et al 1993, 1994), which suggests that the sperm whales (Physeteroidea) are more closely related to the baleen whales (Mysticeti) than to any other Odontoceti species, supported by bootstrap values. In the other eighteen MP analyses, Arnason's hypothesis (Arnason and Gullberg 1994,

1996), which suggests that there are five evolutionary lineages of cetaceans and that there is no particular affinity between the sperm whales and the baleen whales (Arnason and Gullberg 1994, 1996), was sustained (Table 3.3). The largest combined data set (COI+12S+16S+Cytb) produced different results according to the Tv:Ti ratio used in the MP analyses. When Tv:Ti=10:1 and when Tv only were considered, the bootstrap trees favored Arnason's hypothesis with bootstrap values lower than 50 for the monophyletic group of *Physeter* and Balaenopteridae (Figure 3.5.a). However, when Tv:Ti=3:1, Milinkovitch's hypothesis was supported with bootstrap value of 56 (Figure 3.5.b).

Maximum parsimony analyses of the combination of the COI and Cytb gene showed a close relationship between *Hippopotamus* and cetaceans. However, bootstrap values were low (55 and 50, for Tv:Ti=3:1, and 10:1) (trees not shown). MP analyses of the combination of the COI and 12S gene sequences did not support the hippopotamus/cetacean relationship. Contrary to the result with COI+Cytb gene sequences, the bootstrap trees (not shown) supported a ruminant/cetacean clade with bootstrap values of 71, 79 and 65, when Tv where weighted three times and 10 times more than Ti, and when only Tv were considered, respectively. MP bootstrap trees (not shown) of the combination of the results with COI, 12S and Cytb gene sequences also supported a ruminant/cetacean clade with bootstrap values of 63, 66 and 67, for the three Tv:Ti weighting as above, respectively. A ML bootstrap tree (not shown) obtained for the combined COI+12S+Cytb gene sequences also supported a ruminant/cetacean clade (bootstrap value of 52) but the ML bootstrap tree obtained

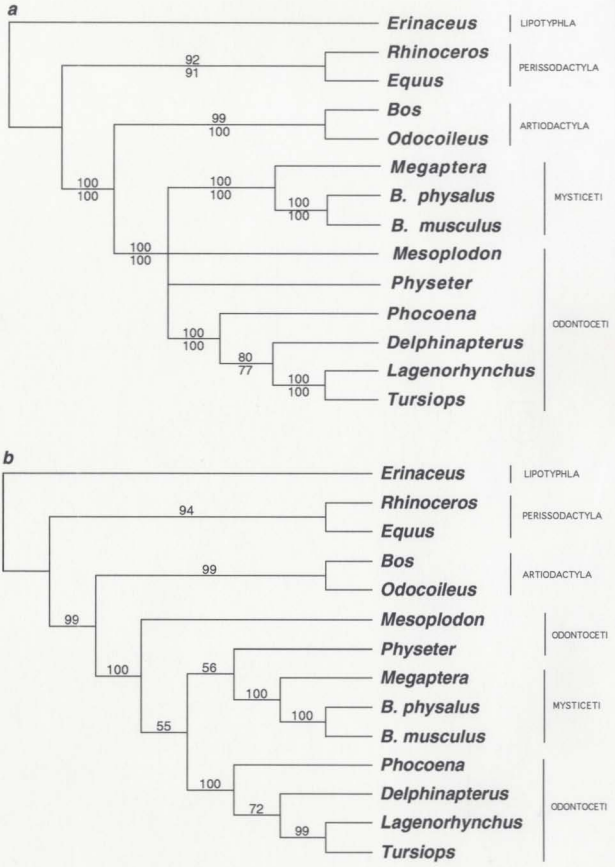
for the combined COI+12S gene sequences did not resolve if either *Hippopotamus* or the Ruminantia species are the closest relatives to Cetacea (trees not shown).

NJ analyses using three different methods (Tamura-Nei, Kimura-2-parameters, and Maximum-likelihood parameters) on the combined data set produced nine bootstrap trees supporting Milinkovitch's hypothesis (9 of 21 analyses). Although, the majority of the NJ analyses on the combined data set gave support to Arnason's hypothesis, Milinkovitch's hypothesis was favored on the largest combined data set (Table 3.3). Figure 3.6 shows the NJ bootstrap tree (1.000 replicates) of maximum likelihood distances (Tv:Ti=3.82: γ=0.195) for the combination of COI+12S+16S+Cytb genes. The monophyletic clade *Physeter*+Balaenopteridae was favored by a bootstrap value of 89 (Table 3.3).

ML analyses supported Arnason's hypothesis on three of the seven combined data sets. When the COI gene sequence was combined with a single other gene (COI+12S, COI+16S, or COI+Cytb), the bootstrap tree obtained favored Arnason's hypothesis. When the COI gene was combined with two or more genes, the bootstrap trees favored Milinkovitch's hypothesis. The ML analysis of the largest data set (COI+12S+16S+Cytb) produced a single tree shown in Figure 3.7. The monophyly of *Physeter* and Balaenopteridae was supported by bootstrap values of 81, with Tv:Ti = 3.82:1, and γ=0.195 (Table 3.3).

Figure 3.5.a. Majority-rule bootstrap consensus tree (heuristic search, maximum parsimony, 300 replicates) based on the 2,548-bp sequences of nine cetaceans, two artiodactyls, and two perissodactyls, using the hedgehog (order Lipotyphla) as outgroup. Numbers indicate bootstrap values. The top values correspond to a MP bootstrap analysis in which Tv were weighted 10 times more than Ti. The bottom values correspond to a MP bootstrap analysis where only Tv were considered. The species corresponding to the genera in this figure are the same indicated in Figure 3.1.

Figure 3.5.b. Majority-rule bootstrap consensus tree (heuristic search, maximum parsimony, 300 replicates) based on the 2,548-bp sequences of the same species listed in Figure 3.5.a. Numbers indicate bootstrap values corresponding to a MP in which Tv were weighted three times more than Ti.


a
Erinaceus
Rhinoceros
Equus
Bos
Odocoileus
Megaptera
B. physalus
B. musculus
Mesoplodon
Physeter
Phocoena
Delphinapterus
Lagenorhynchus
Tursiops
LIPOTYPHLA
PERISSODACTYLA
ARTIODACTYLA
MYSTICETI
ODONTOCETI
92
91
99
100
100
100
100
100
100
100
100
100
80
77
100
100
b
Erinaceus
Rhinoceros
Equus
Bos
Odocoileus
Mesoplodon
Physeter
Megaptera
B. physalus
B. musculus
Phocoena
Delphinapterus
Lagenorhynchus
Tursiops
LIPOTYPHLA
PERISSODACTYLA
ARTIODACTYLA
ODONTOCETI
MYSTICETI
ODONTOCETI
94
99
99
100
56
100
100
55
100
72
99

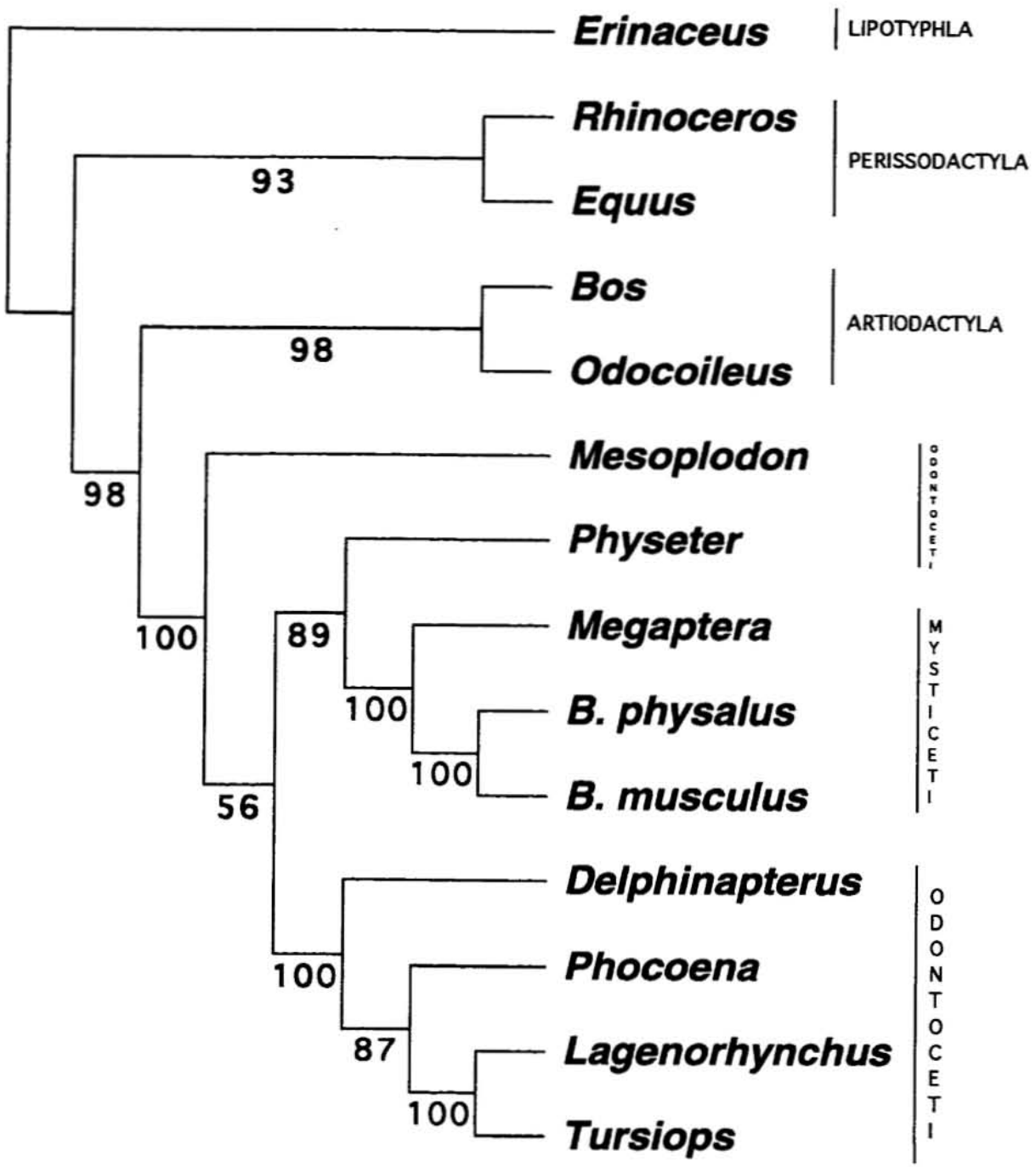
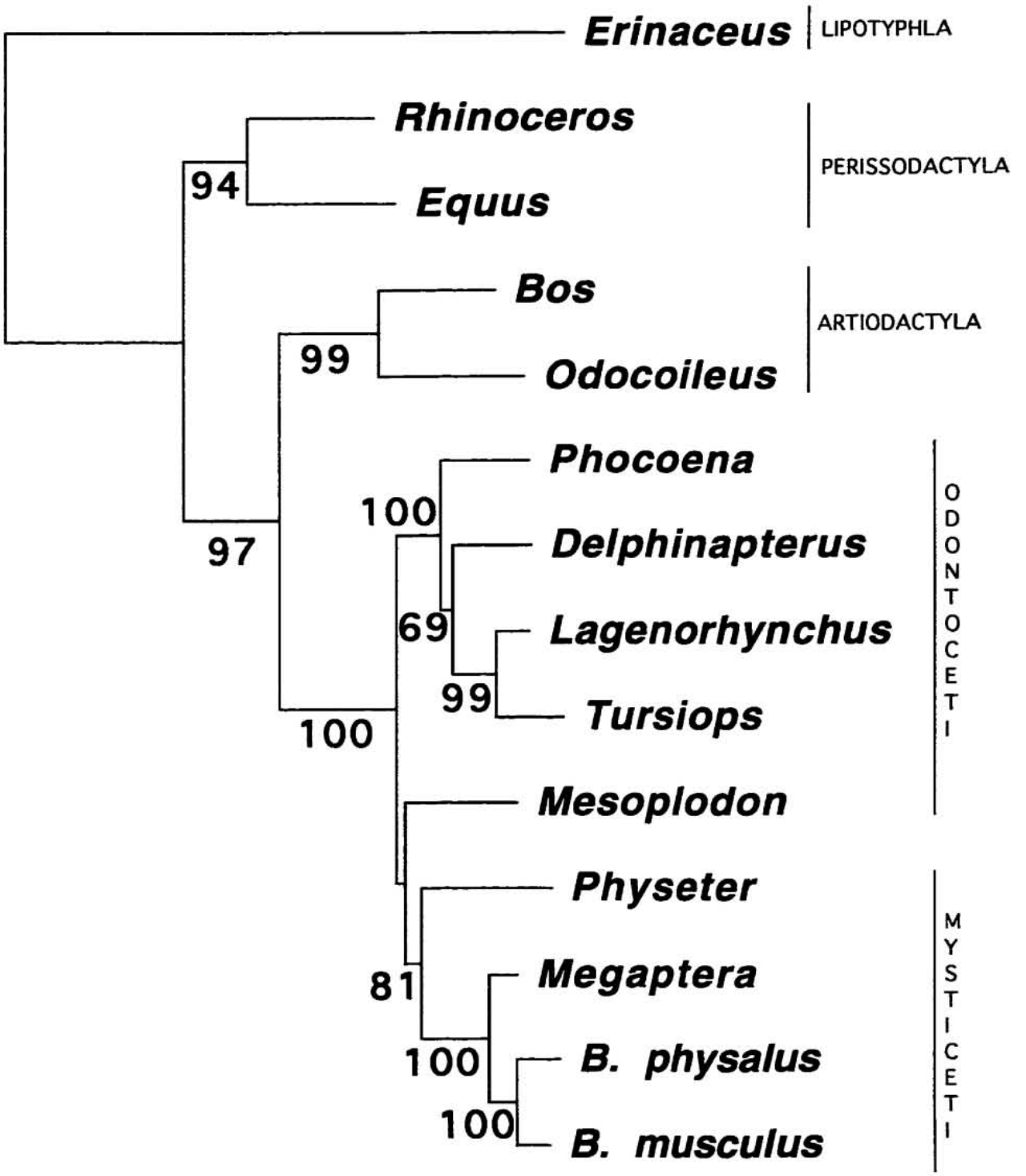

Erinaceus
LIPOTYPHLA
Rhinoceros
Equus
PERISSODACTYLA
93
Bos
Odocoileus
ARTIODACTYLA
98
Mesoplodon
Physeter
ODONTOCETI
98
100
89
Megaptera
B. physalus
B. musculus
MYSTICETI
100
100
56
Delphinapterus
Phocoena
Lagenorhynchus
Tursiops
ODONTOCETI
100
87
100

Figure 3.7. Maximum-likelihood tree based on the 2.548-bp sequences of the same species listed in Figure 3.5.a. Numbers indicate bootstrap values (300 replicates). Transversions were weighted 3.82 times more than transitions. ratio estimated previously by heuristic search and likelihood options. The gamma shape distribution parameter was γ=0.195. value also estimated by the same heuristic search. The species corresponding to the genera in this figure are the same indicated in Figure 3.1.


Erinaceus
LIPOTYPHLA
Rhinoceros
Equus
94
PERISSODACTYLA
Bos
Odocoileus
99
ARTIODACTYLA
Phocoena
100
Delphinapterus
97
69
Lagenorhynchus
99
Tursiops
100
ODONTOCETI
Mesoplodon
Physeter
81
Megaptera
100
B. physalus
100
B. musculus
MYSTICETI

3.4. DISCUSSION

3.4.1. Monophyly of cetaceans

Cetaceans are a monophyletic group, irrespective of the kind of phylogenetic analysis (maximum parsimony, neighbor-joining, and maximum-likelihood) or of the gene sequences combinations used to perform in the present study. The monophyly of cetaceans was previously observed in molecular phylogenies, using the 12S and 16S genes (Milinkovitch et al. 1993) in combination with the Cytb gene (Milinkovitch et al. 1994, 1995), in complete Cytb sequences (Arnason and Gullberg 1994, 1996; Milinkovitch et al. 1996), and in a recent morphological study (Heyning 1997).

The degree of adaptation of whales and dolphins to underwater life is unique among mammals. The cetaceans as well as the manatees and dugongs (order Sirenia), are the only mammals that have become true marine mammals. Heyning (1997) identified at least 17 synapomorphies of all cetaceans when compared to members of the orders Artiodactyla, Perissodactyla, and Proboscidea. Some of the synapomorphies of cetaceans in relation to these mammalian orders are: a complete absence of hind limbs, front limbs modified into flippers lacking movement except at shoulder joint, the presence of blubber, and elongate rostrum with mesorostral gutter, and the presence of a melon (Heyning 1997).

3.4.2. Phylogenetic relationships between Cetacea and Artiodactyla

In all the phylogenetic analyses performed in the present study, the cetaceans were more closely related to the artiodactyls than to the perissodactyls. This sister relationship between Cetacea and Artiodactyla is in agreement with morphological studies (Novacek 1992), palaentological findings (Gingerich et al. 1990, 1994; Thewissen and Hussain 1993; Thewissen et al. 1994) and previous molecular studies (Irwin et al. 1991; Milinkovitch et al. 1993, 1994; Graur and Higgins 1994; Arnason and Gullberg 1996; Montgelard et al. 1997).

The maximum parsimony analyses of the COI and COI+Cytb gene sequences suggests that the *Hippopotamus amphibius* is the artiodactyl most closely related to cetaceans. This close relationship between the cetaceans and the hippos might be so strong that Irwin and Arnason (1994) based on phylogenetic analyses of complete cytochrome b gene sequences suggested that the genus *Hippopotamus* is more closely related to Cetacea than to members of the other suborders of Artiodactyla, which would make the order Artiodactyla paraphyletic. Other recent molecular studies based on mitochondrial genes (Arnason and Gullberg 1996; Hasegawa et al. 1997; Montgelard et al. 1997), the nuclear casein gene (Gatesy et al. 1996), and the gene for the blood-clotting protein γ-fibrinogen (Gatesy 1997), also supported a Cetacea/Hippopotamidae clade.

If the Hippopotamidae species are the closest relatives to extant cetaceans, as suggested by the COI MP analyses, then the shared aquatic specializations of these two groups of mammals can be interpreted as synapomorphies, due to descent from a

common ancestor. Several potential synapomorphies of cetaceans plus hippos that could support the molecular data were suggested by Gatesy (1997), including the lack of sebaceous glands, and the absence of hair (Ling 1974), the lack of scrotal testes (Erken et al., 1994), and the nursing of offspring underwater (shared by *Hippopotamus amphibius* and extant cetaceans)(Slijper 1962).

Although there are numerous similarities between fossil teeth of primitive cetaceans and mesonychian ungulates (Thewissen et al. 1994), there is no fossil evidence for a common ancestor between cetaceans and hippos, and evolutionary convergences are considered to be responsible for the similiarities of aquatic specializations between cetaceans and hippos (Gatesy 1997). However, based on MP and NJ analyses of the complete Cytb and 12S sequences Montgelard et al. (1997) calculated that the divergence between Cetacea and Hippopotamidae (Ancodonta) occurred 53 million of years ago (Mya). Their suggestion was inconsistent with the fossil record, since the oldest fossil of a cetacean (*Pakicetus inachus*) is known to be from 52 Mya (Thewissen and Hussain 1993; Thewissen et al. 1994).

MP analyses of the COI+12S and COI+12S+Cytb combined gene sequences did not support a Hippopotomidae/Cetacea clade. On the contrary, these analyses gave support for a Ruminantia/Cetacea monophyly, which would also make the order Artiodactyla paraphyletic. These results are not congruent with Montgelard et al. (1997), but on the other hand, they are in agreement with Graur and Higgins (1994) MP and NJ analyses of 11 nuclear-encoded protein gene sequences and five mitochondrial gene sequences (Cytb, 12S, ATPase 6, NADH-1, and four-combined

tRNAs), which suggested that members of the suborder Ruminantia (cows, deers, giraffes, goats, and others) are more closely related to cetaceans than to members of the two other suborders of Artiodactyla, Suiformes (pigs, peccaries, and hippopotamus) and Tylopoda (camels and llamas). These authors estimated that Cetacea and Ruminantia diverged from each other 45-49 Mya. This assumption is somewhat more recent than the supposed divergence between Suiformes and Artiodactyla (~55-60 Mya) and Tylopoda and Artiodactyla (>45 Mya) (Webb and Taylor 1980).

Both the Hippopotamidae/Cetacea clade (Irwin and Arnason 1994) hypothesis and the Ruminantia/Cetacea clade (Graur and Higgins 1994) hypotheses were tested by Hasegawa and Adachi (1996) using maximum-likelihood methods for phylogenetic analyses of several mitochondrial gene sequences (Cytb, 12S, ATPase 6, NADH dehydrogenase I, and four-combined tRNAs). They concluded that "none of the proposed hypotheses was convincingly supported by the existing sequence data when analyzed carefully by the ML method".

The results of MP, NJ, and MP analyses in the present study did not favor either of the hypotheses. Different combinations of gene sequences generated different results. Based on them, this study is in agreement with Hasegawa and Adachi (1996) that more molecular data must be obtained and phylogenetic methods must be improved in order to obtain a confident molecular phylogeny able to resolve the relationships between Cetacea and suborders of Artiodactyla. In addition, the

paleontological and morphological data must also be considered for reconstruction of a true Cetacea/Artiodactyla phylogeny.

3.4.3. The placement of Physeteridae within Cetacea

The phylogenetic analyses performed in this study yielded different answers in relation to the placement of the sperm whales within Cetacea, depending on the combination of gene sequences and the method of analysis used. When the COI sequences were analyzed alone, a sister relationship between the sperm whales (*Physeter*) and the mysticetes (Balaenopteridae) was not identified (Figures 3.2, 3.3, 3.4). All analyses (MP, NJ, and ML) favored Arnason's hypothesis (Arnason and Gullberg 1994, 1996) and were contrary to Milinkovitch's hypothesis (Milinkovitch et al. 1993, 1994). When the COI gene sequences were combined with one of the three other mitochondrial genes (12S, 16S, Cytb) sequences, all the phylogenetic analyses of the possible combined data sets also favored Arnason's hypothesis. However, when the COI sequences where combined with two or more gene sequences the results sometimes favored Arnason's and sometimes favored Milinkovitch's hypothesis, depending of the type of analysis performed (Table 3.3).

Milinkovitch (1995) presented some morphological evidence for his hypothesis of a close relationship between sperm whales and baleen whales, based on DNA sequences of fragments of the 12S, 16S, and Cytb genes (Milinkovitch et al. 1993, 1994). A new morphological character was proposed by Milinkovitch (1995), "the number of nasal passages distal to the bone nares", with two states: two nasal

passages (ancestral state), present in baleen whales and sperm whales, and a single nasal passage (derived state) present in all odontocetes but not in the sperm whales. The presence of two nasal passages was considered a synapomorphy of baleen and sperm whales by this author. However, Heyning (1989, 1997) did not agree with Milinkovitch's assumption and coded this feature as two characters: number of blowholes and confluence of nasal passages. An intermediate state (single blowhole, nasal passages not confluent) present only in Physeteridae was proposed by Heyning (1997).

Another morphological character pointed out by Milinkovitch (1995) as supporting his hypothesis was the presence of a "vestigial melon (the atrophied remnant of an acoustically functioning melon)" in mysticetes. This small fatty structure in the same relative position as the melon of odontocetes was described by Heyning and Mead (1990) who hypothesized that its original function was to allow the blowholes to open smoothly with the contraction of the nasal plug. Milinkovitch's assumption that this fatty structure is a "vestigial melon", regressed from a larger melon in an ancestor, should not be considered according to Heyning (1997) because Milinkovitch (1995) did not provide any evidence that supported his idea. In his cladistic analyses, Heyning (1997) identified thirteen characters that support the Odontoceti monophyly (including the family Physeteridae) with stong statistical support (CI= 0.92 ; RI=0.96).

The supposition of Milinkovitch et al. (1993) that the common ancestor of baleen whales and sperm whales might have lived between 10 and 15 million years

ago (Mya) was observed to be erroneous by Arnason and Gullberg (1994) who showed that the separation between the lineages of the family Balaenidae (right whales) and the other three families of Mysticeti (Balaenopteridae, Neobalaenidae, and Eschrichtiidae) occurred more than 17 Mya, based on an interpretation of the paleontological record of Mysticeti (Barnes and McLeod, 1984; Barnes et al., 1985). The oldest Physeteridae fossils from Early Miocene (~20 Mya) deposits in Patagonia, Argentina (Barnes et al., 1985) also do not support the separation proposed by Milinkovitch et al. (1993). The relative rate of evolution implied by restriction enzyme maps of fourteen cetacean species (Ohland et al. 1995) was also concordant with the fossil-based phylogenies (Fordyce 1980; Barnes et al. 1985) and not concordant with Milinkovitch et al. (1993, 1994).

If Milinkovitch's hypothesis were true and Mysticeti and Physeteridae are sister groups, then they must have shared a common ancestror during their evolution. A consequence of this assumption is that the Mysticeti first evolved the Physeteridae characteristics and later lost them, in order to develop a different feeding strategy, which implied loss of teeth, loss of echolocation, loss of spermaceti organ and development of baleen plates (Ohland et al. 1995). This evolutionary scenario seems to be less parsimonious then the assumption that the Mysticeti evolved from an older toothed whale ancestor than from a common ancestor with the Physeteridae.

The MP analysis of the Cytb gene by Arnason and Gullberg (1996) produced an unresolved bootstrap tree with five major lineages of cetaceans. The results obtained using the COI gene sequence alone and the COI gene sequence combined

with only one of the three other mitochondrial gene sequences are in agreement with Arnason's hypothesis. However, the analyses when more than two gene sequences were combined favored Milinkovitch's hypothesis, particularly when the NJ and ML methods were used (Table 3.3).

3.4.4. Monophyletic groups within Cetacea

Four major groups of cetaceans, corresponding to the taxonomic groups Physeteridae, Ziphiidae, Delphinida, and Mysticeti, were identified by the phylogenetic analyses of the COI gene sequences. However, phylogenetic relationships among the four groups could not be resolved by MP, NJ, or ML analyses. Five clades of extant cetaceans (the four obtained here plus Platanistidae) were identified by Arnason and Gullberg (1996) based on MP analyses of the complete Cytb sequences of 28 cetaceans. These authors also concluded that the rates of molecular evolution of the Cytb gene of the five groups were similar, based on the lack of bootstrap support to resolve the relationships among them.

All phylogenetic analyses of the COI sequences including all possible combination of gene sequences identified the Balaenopteridae (Mysticeti) as a monophyletic group, independent of the method used (MP, NJ, ML). The suborder Mysticeti was recognized as a monophyletic group by all previous molecular studies using mitochondrial genes (Milinkovitch et al. 1993, 1994, 1996; Arnason and Gullberg 1994, 1996; Hasegawa et al. 1997). The three *Balaenoptera* species (*B. acutorostrata, B. musculus, and B. physalus*) constituted a monophyletic group, but

the relationships among them were undetermined by MP analyses of the COI sequences (Figure 3.2), while NJ analyses (Figure 3.3) and ML analyses (Figure 3.4) produced two different combinations of relationships between these species. Strong support was obtained for a sister relationship between the humpback whale (*M. novaeangliae*) and the other baleen whales of the genus *Balaenoptera* in all the phylogenetic analyses of the COI gene sequence alone and the combined gene sequence data sets. Relationships between *B. musculus*, *B. physalus*, *M. novaeangliae*, and *E. robustus* (Eschrichtiidae) were also undetermined by the MP analyses of Cytb gene sequences of Arnason and Gullberg (1994).

The phylogenetic position of the beaked whales (Ziphiidae), represented here by the genus *Mesoplodon*, was undetermined by any of the COI gene sequence analyses, or by the majority of the analyses performed on the combined gene sequence data sets. When there was bootstrap support for the placement of the Ziphiidae as an outgroup of the other three cetacean clades (such as, in Figures 3.5.b and 3.6), bootstrap values were very low. All previous molecular studies based on mitochondrial gene sequences (Milinkovitch et al. 1993, 1994, 1996; Arnason and Gullberg 1994, 1996; Hasegawa et al. 1997) failed to determine the place of Ziphiidae within Cetacea with bootstrap support. The uncertain placement of Ziphiidae was also observed in some morphological studies (Barnes 1984; Heyning 1989), although a recent cladistic analysis of morphological characters placed the Ziphiidae between the Physeteridae and the other Odontoceti families (Heyning 1997).

Strong support was found for the monophyly of the family Delphinidae by all the phylogenetic analyses of the COI gene sequences (Figures 3.2, 3.3, 3.4). Within the Delphinidae, the relationships among species were not well-resolved with the exception of a monophyletic group composed by four species (*D. delphis*, *L. albirostris*, *S. frontalis*, *T. truncatus*) that was well-supported by MP (Figure 3.2) and NJ analyses (Figure 3.3) of COI gene sequences. A recent molecular study of the family Delphinidae based on Cytb sequences (LeDuc 1997) was the first to resolve the phylogenetic relationships among species within this family that were not resolved by previous studies (Milinkovitch et al. 1994, Arnason and Gullberg 1996, Hasegawa et al. 1997).

MP, NJ, and ML analyses of the COI gene sequence alone (with the exception of the MP analysis when Tv:Ti=3:1) and MP, NJ, and ML analyses of all the combined gene sequence data sets (Figures 3.2 to 3.7) supported the monophyly of the superfamily Delphinoidea, which includes the families Delphinidae (dolphins), Phocoenidae (porpoises), and Monodontidae (white whales). A Delphinoidea monophyletic group was previously detected by molecular studies (Milinkovitch et al. 1994; Arnason and Gullberg 1996; Hasegawa et al. 1997).

A sister relationship between Pontoporidae (represented by *P. blainvillei*) and Delphinoidea was supported by MP and ML bootstrap analyses of COI gene sequences. This result is in agreement with previous molecular phylogenies that included the same species (Arnason and Gullberg 1996; Hasegawa et al. 1997).

3.4.5. COI alone *versus* COI combined with different mitochondrial genes

Why do different combinations of mitochondrial gene sequences suggest different phylogenies for cetaceans, if there is only one historical reality ?

Mitochondrial DNA sequences are considered to be a good molecular tool for inference of evolutionary relationships among mammals because all mitochondrial genes are inherited together without recombination and there is no confusion of orthologous and paralogous genes (Cao et al. 1994; Simon et al. 1994; Honeycutt et al. 1995; Russo et al. 1996). However, it has been observed that different mitochondrial genes can generate different phylogenies for the same group of organisms (Goodman et al. 1982, Hedges 1994; Russo et al. 1996). Combinations of different mitochondrial gene sequences should reflect the evolution of a single genetic linkage group with the same phylogenetic history (Vogler and Welsh 1997).

The difference between phylogenies obtained in this study by the analyses of the COI gene sequence and by the combination of the COI gene sequence with sequences of different genes may be a consequence of the sampling properties of DNA sequence data in phylogenetic analysis, a problem that was investigated by Cummings et al. (1995).

The assumption that increasing the quantity of DNA sequence data will improve the phylogenetic estimation of evolutionary trees is widespread (Churchill et al. 1992; Huelsenberg and Hillis 1993; Hasegawa et al.1997). The assumption is that the historical signal will rise above misleading noise as more sequence is added

(Naylor and Brown 1997). If this assumption were true, the most informative sequence set will combine the 495-bp fragment of COI gene sequence with the complete cytochrome b gene sequence (1,140-bp) and sequence fragments of the 12S and 16S genes (913-bp), which together represent approximately 20% of the total mitochondrial genome (2,548 of 12,234-bp) (Table 3.3). The results of the analyses of the 2,548-bp sequences agree with Milinkovitch's hypothesis, with the exception of the MP analyses where Tv:Ti = 10:1 and Tv only were considered (Figures 3.5, 3.6, 3.7).

However, Naylor and Brown (1997) showed a particular example where increasing of amount of DNA sequence of mitochondrial genes may not represent accurately the evolution of the whole mitochondrial DNA. Their analysis used a phylogenetic parsimony analysis of the entire protein-coding portion of the mitochondrial genome for a "well-accepted phylogeny". These authors did not obtain the "expected phylogeny" and achieved bootstrap support for incorrect placements of taxa.

The phylogenetic analyses (MP, NJ, and ML) of the COI gene sequence alone in this study, and the MP and NJ analyses of the cytochrome b gene sequence alone (Arnason and Gullberg 1994, 1996) did not support Milinkovitch's hypothesis, as well as the phylogenetic analyses of the COI gene sequence combined with a single gene sequence (12S, 16S, or Cytb). On the other hand, MP analyses and ML analyses of the cytochrome b gene sequences (Milinkovitch et al. 1995, 1996; Hasegawa et al. 1997) supported Milinkovitch's hypothesis. The MP analyses performed by

Milinkovitch et al. (1996) based on the cytochrome b gene sequences suggested that character weighting and species sampling influenced the phylogenies obtained for the cetaceans.

The efficiencies of different mitochondrial genes in recovering a known phylogeny were evaluated by Russo et al. (1996). Among the thirteen genes analyzed, the "best genes" (the ones that produced the 'correct tree' in all tree-building methods or algorithms for both amino acid and nucleotide sequence data) were the NADH-4, NADH-5, and cytochrome b genes. The COI gene showed a relatively good performance but nucleotide sequences sometimes produced incorrect trees, probably due to its small extent of sequence divergence (Russo et al. 1996). Naylor and Brown (1997) also analyzed the efficiencies of mitochondrial genes to estimate a known phylogeny, superimposing the sequence data onto the accepted tree and measuring how well each site fits the tree. They observed that the "best genes" were the ATPase 6 and the NADH-4L genes, and that the COI and the cytochrome b genes were among the intermediate efficient genes. Among the "best genes" proposed by Russo et al. (1996) and by Naylor and Brown (1997) only the cytochrome b has been gene used to study phylogenetic relationships among cetaceans.

Species sampling has a major impact on phylogenetic inference according to Lecointre et al. (1993). The present study presents the largest nucleotide data set ever assembled for the order Cetacea: 2,548 bp (COI+12S+16S+Cytb) versus 1,140bp (complete cytochrome b) of Arnason and Gullberg (1996) and Hasegawa et al. (1997), and 1,352bp (12S+16S+Cytb) of Milinkovitch et al. (1994). However, the number of

taxa presented here for the COI+12S+16S+Cytb data set (nine cetaceans + five non-cetaceans) is smaller than that used by Milinkovitch et al. (1994) (21 cetaceans + three non-cetaceans), Arnason and Gullberg (1996) (28 cetaceans + 12 non-cetaceans) and Hasegawa et al. (1997) (27 cetaceans + 13 non cetaceans). The use of larger sequence data sets for construction of trees in which few taxa are used to represent strategic taxonomic groups has been extensively used (e.g. Meyer and Wilson 1990), however, the results and conclusions obtained could be different with the addition of more representatives of the sampled groups as was shown by Lecointre et al. (1993).

The use of fewer nucleotides per species and more species representing more taxa of the groups studied was recommended by Lecointre et al. (1993), because the impact of species sampling may be stronger than the impact of sequence variation. If this is correct, the analyses of the sequence data set for the COI gene (16 cetaceans + seven non-cetaceans) may be more powerful than the analyses for the largest data set (nine cetaceans + five non-cetaceans).

In summary, the different phylogenies obtained in this study, according to the different genes (or combination of genes) used, may be a consequence of the sampling properties of DNA sequence data, of the efficiencies of different genes in recovering phylogenies, and of the impact of species sampling in phylogenetic inference.

3.4.6. MP *versus* NJ *versus* ML in Cetacea

Why do different phylogenetic methods suggest different phylogenies for cetaceans, if there is only one historical reality ?

Bootstrapping was the method used in this study for testing the confidence of the phylogenetic results and the robustness of the trees obtained. Bootstrap values have been extensively used as indicators of support for a monophyletic group since Felsenstein (1985) proposed to use bootstrapping to estimate confidence limits of internal branches in phylogenetics analyses of DNA sequences (Milinkovitch et al. 1996). Bootstrapping is a random resampling of the data set with replacement. Bootstrap estimates are evaluated by counting the number of times that each grouping of taxa occurs among the numerous replicates (Lecointre et al. 1993; Swofford et al. 1996). A bootstrap consensus tree contains all the major grouping of species (or nodes) and the supported nodes are those with bootstrap values superior to 50% in the tree (Lecointre et al. 1993). Three different tree-building methods were used in this study to obtain bootstrap consensus trees: maximum parsimony, neighbor-joining, and maximum-likelihood.

The majority of the maximum parsimony (Swofford 1993) analyses performed in the present study identified four clades of cetaceans (favoring Arnason's hypothesis) , but did not resolve the relationships among them (Table 3.3). The only exceptions were the MP analyses for the COI+16S+Cytb sequences (tree not shown) and for the COI+12S+16S+Cytb combined gene sequences (Figure 3.6), which supported Milinkovitch's hypothesis. Maximum parsimony analysis has been the method used most extensively to infer molecular phylogenies (Swofford et al. 1996). In this type of analysis, the most-parsimonious tree is the one that requires the smallest number of evolutionary changes to explain the differences among taxa

(Avise 1994). The parsimony method for DNA sequence works by selecting trees that minimize the total length, or the number of tranformations from one character state to another (steps) necessary to explain a given set of data (Swofford et al. 1996).

Maximum parsimony analyses performed in this study were done with three different ratios of Tv:Ti (3:1, 10:1, 1:0) to assess the effect of different rates of evolution between Tv and Ti. Milinkovitch et al. (1995) observed that because Arnason and Gullberg (1994) used a weighting scheme based on codon position only and considered transitions and transversions to be equally informative, their analyses could lead to erroneous results. Milinkovitch et al. (1993, 1994, 1995, 1996) used MP analyses with different Tv:Ti ratios in their molecular studies that showed a close relationship between sperm whales and baleen whales. Transition substitutions accumulate more quickly over time than transversions, which increases sequence divergence, creates noise, and hides the phylogenetic signal (De Salle et al. 1987; Meyer and Wilson 1990; Irwin et al. 1991; Hillis et al. 1994). The ratio of transversions to transitions (Tv:Ti) was not a major factor in the phylogenetic analyses performed here since in the majority of the analyses Arnason's hypothesis was supported. Only in two of 24 cases, were bootstrap trees supporting Milinkovitch's hypothesis obtained. In both cases the ratio Tv:Ti was 3:1. This ratio presents the highest sequence divergence and noise among the Tv:Ti ratios used in this study. These results are in agreement with Milinkovitch et al. (1996) who observed that different weighting schemes produced results very similar to those

yielded by the unweighted searches. when informative taxa were included in the cytochrome b gene analyses of cetaceans together with one or two outgroups.

The neighbor joining method (Saitou and Nei 1987) yielded different bootstrap trees according to which gene or combination of genes was analyzed and the method used to calculate the distance matrices (Table 3.3). All the bootstrap results of the NJ analyses for the COI gene sequence alone or in combination with a single other gene sequence favored Arnason's hypothesis. They were in agreement with the MP analyses for the same data sets. Arnason and Gullberg (1994, 1996) did not use the neighbor joining method for calculating distance matrices, but they examined the distance, in terms of the percent sequence difference, within and among cetacean clades. Their results were consistent with their MP analyses of the cytochrome b gene. However, NJ trees produced from the maximum-likelihood distance matrices for the combined 12S+16S+Cytb gene sequences using PHYLIP (Felsenstein 1993) supported a sister relationship between sperm whales and baleen whales (Milinkovitch et al. 1994). All the NJ analyses performed here using maximum-likelihood distance matrix parameters for the COI gene sequence combined with two or more genes (12S, 16S, Cytb) agreed with Milinkovitch's hypothesis.

When the maximum-likelihood method was used to bootstrap the tree with the maximum-likelihood ratio (Felsenstein 1981), using PAUP (Swofford 1997), different results were obtained according to which set of sequence data was used. According to Hasegawa et al. (1991), the ML method is known as the most efficient

method to use in combined sequence data sets because it decreases the error due to different evolutionary rates among genes. The results were essentially similar to the ones obtained using NJ methods. The ML bootstrap trees for the COI sequence alone or combined with a single gene favored Arnason's hypothesis, but the ML bootstrap trees for the COI combined with two or more genes supported Milinkovitch's hypothesis (Table 3.3). Milinkovitch et al. (1993, 1994, 1996) and Hasegawa et al. (1997) used the ML method in their analyses of the cytochrome b gene sequence alone or combined with the 12S and 16S gene sequences, but Arnason and Gullberg (1994, 1996) did not use this method in their studies using the cytochrome b gene. According to Felsenstein (1981), the maximum-likelihood method is superior to the other methods (MP, NJ) for achieving the correct phylogeny when rates of evolution differ among lineages. The ML method was also found to be the most efficient when sequences of different genes were used in combination because it decreases the errors due to different evolutionary rates among genes (Hasegawa et al. 1991, 1997).

In general, MP analyses produced different phylogenies than NJ and ML analyses, when combination of three or more genes were used. These results suggest that molecular phylogenies for cetaceans are sensitive to the different methods of phylogenetic analysis, in addition to the combination of different mitochondrial genes used in the analyses.

If different methods of phylogenetic analysis yielded different results, which method is most efficient ?

The efficiency of five methods of phylogenetic analysis for a four-taxon tree with equal rates of evolution was investigated by Hillis et al. (1994). Weighted parsimony (any weighting of transversions over transitions from 5:1 to infinity) required ten times more nucleotides to achieve the same performance as unweighted parsimony, and 250 times more nucleotides than NJ with Kimura distance parameters. Given this result, the MP analyses where Tv:Ti = 10:1 performed here, which supported the Arnason's hypothesis, should be considered more efficient than the MP where Tv:Ti = 3:1 and the NJ analyses using Kimura parameters. However, the use of only four taxa in phylogenetic analyses (as was done by Hillis et al. 1994) is not recommended because the impact of species sampling on bootstrap results has been observed to be strong in 4-species trees (Lecointre et al. 1993).

The relative efficiencies of four different methods of analysis in recovering a known vertebrate phylogeny was evaluated by Russo et al. (1996). They observed that among the tree-building methods tested (MP, NJ, ML, and minimum evolution), NJ tended to show small d_T's (topological distances of reconstructed trees form true tree), whereas ML tended to show large d_T's. However, these authors concluded that the efficiencies of the four methods in obtaining the 'correct tree' were approximately the same. The use of a "good gene" or a large data set of nucleotide sequence or amino acid sequence seemed to be more important than the choice of the tree-building method (Russo et al., 1996).

3.5. Conclusions

According to the phylogenetic analyses performed in this study, the order Cetacea is monophyletic and the cetaceans are more closely related to the Artiodactyla than to the Perissodactyla. Four major clades were identified among cetaceans with different taxonomic rankings (Physeteridae, Ziphiidae, Delphinida, and Mysticeti), but the relationships among these four groups were not resolved.

The results achieved in the present study suggest that molecular phylogenies for cetaceans are sensitive to different methods of phylogenetic analyses and to combination of different mitochondrial genes. MP analyses produced different phylogenies than NJ and ML analyses, when combinations of three or more genes were used. NJ, using ML parameters, yielded the same results as ML analyses alone.

Based on the results obtained in this study and on the arguments presented previously, I do not agree with Milinkovitch's proposed reevaluation of the taxonomic classification and reinterpretation of the morphological, physiological and behavioral evolution of cetaceans (Milinkovitch et al. 1993, 1994; Milinkovitch 1995) and rejection of the traditional view of toothed-whale monophyly (Hasegawa et al. 1997). Although these authors found some molecular evidence for close relationships between baleen whales and sperm whales, based on phylogenetic analyses of sequence fragments of three mitochondrial genes, their results were not supported by this study or by other studies using mitochondrial genes (Arnason and Gullberg 1994, 1996).

For a reevaluation and possible modification of the traditional taxonomic classification of the order Cetacea it will be necessary to combine molecular, morphological and paleontological evidence. Phylogenetic analyses performed by Milinkovitch et al. (1993, 1994) were weakly supported by low bootstrap values. His attempt to provide morphological evidence to support a Mysticeti/Physeteridae clade (Milinkovitch 1995) was strongly criticized by Heyning (1997), who performed a cladistic analysis of a large number of morphological characters that strongly supported the monophyly of odontocetes, including Physeteridae. Fossil records to support the existence of a common ancestor of baleen whales and sperm whales have never been found (Thewissen and Hussain 1993; Thewissen et al. 1994).

CHAPTER 4

PHYLOGENETIC RELATIONSHIPS OF ANGEL SHARKS (Squatinidae, Elasmobranchii) FROM SOUTHERN BRAZIL AS SUGGESTED BY THE MITOCHONDRIAL CYTOCHROME B GENE

4.1. Introduction

The family Squatinidae, angel sharks, comprises a single genus that includes fifteen extant species. These species share a number of synapomorphic characters, including a flattened body, a ray-like shape and a terminal mouth. Angel sharks are considered to be intermediate in form between sharks and rays because of their ray-like body shape (Compagno 1984; Boeckmann 1996).

Three species of the genus *Squatina* (Dumeril 1806) are endemic to the continental shelf of southeastern South America, between latitudes 24°00'S and 42°00'S (Figure 4.1): *Squatina argentina* (Marini 1930), *S. guggenheim* (Marini 1936) and *S. occulta* (Vooren and Silva 1991). Fisheries for angel sharks are of great economic importance in Rio Grande do Sul State, Brazil. An average catch of 1.000 metric tons/year has been recorded during the 80's, with maximum catch of 2.500 metric tons/year in 1988 (Boeckmann 1996).

These species share a number of synapomorphies such as the smooth-edged anterior nasal appendages without fringes (an appendix to the internal face of the nasal barbels) and a simple skin fold bordering the head anterolaterally without lobes. They differ in tooth formula, shape and relative size of the pectoral fin, and presence

Figure 4.1. Map of South America, showing the area of occurrence (in gray) of the three species of angel sharks (*Squatina argentina*, *S. guggenheim* and *S. occulta*), and the range of fishing vessels that provided tissue samples of these species (in dark gray). Samples were collected in Rio Grande, Rio Grande do Sul State, Brazil.


80°W
60°W
40°W
0°
20°S
40°S
BRAZIL
Paraguay
Rio Grande
do Sul State
Rio Grande
Argentina
Uruguay

or absence of a dorsal row of spines. They also differ in their vertical distribution in the water column, feeding behavior, fecundity, body size at birth and at first maturity, and maximum size (Compagno, 1984; Vooren and Silva, 1991).

Vooren and Silva (1991) recently described the species *S. occulta* which differs from *S. argentina* by the shape and relative size of the pectoral fin, from *S. guggenheim* by the lack of a dorsal row of spines, and from both species by the tooth formula and color of the dorsal body surface. Before the description of *S. occulta* and the re-description of *S. guggenheim* by Vooren and Silva (1991), only one species of *Squatina* was thought to occur in the southern coast of South America (Figueiredo 1977; Compagno 1984), whereas *S. occulta* and *S. guggenheim* were misidentified as *S. argentina* in some studies (e.g. Cousseau 1973; Rahn and Yesaki 1976).

Few genetic studies have been performed on the family Squatinidae. Solé-Cava et al. (1983) and Solé-Cava and Levy (1987) examined allozymic differences among three morphotypes of the putative species *Squatina argentina* in southern Brazil, and concluded that they represented three reproductively isolated species. These studies were essential for the description of the new species *Squatina occulta*, and for the re-description of *S. argentina* and *S. guggenheim* by Vooren and Silva (1991). A study of forty enzymes encoded by 72 presumptive gene loci in the Pacific angel shark, *Squatina californica*, provided a baseline description of elasmobranch gene expression for comparative studies of other species of sharks and rays (Gaida 1995).

Elasmobranchs (sharks and rays) are excellent organisms for the study of molecular evolution in vertebrates because there is an abundant stratigraphic record available for this group (Maisey 1984; Cappetta 1987), which permits accurate calibration of rates of DNA and protein sequence evolution (Martin and Palumbi 1993). The rate of mitochondrial evolution in sharks is seven to eight-fold slower than the rate in mammals as measured by nucleotide substitution in the cytochrome b and cytochrome oxidase genes (Martin et al. 1992). Molecular phylogenetic relationships within the subclass Elasmobranchii (class Chondrichthyes) have been studied with the mitochondrial cytochrome b gene (Martin and Palumbi 1993; Martin 1995; Kitamura et al. 1996), the 12S mitochondrial gene (Dunn and Morrissey 1995), the nuclear small ribosomal subunit (18S rRNA) gene combined with a fragment of the mitochondrial cytochrome b gene (Bernardi and Powers 1992), and the cytochrome b gene in combination with the NADH-2 gene (Naylor et al. 1997).

Phylogenetic analyses based on the complete cytochrome b gene (1,146bp) of 13 species of sharks indicated that the family Carcharhinidae (order Cacharhiniformes) is monophyletic, as well as the family Lamnidae (order Lamniformes), and that the genus *Heterodontus* (order Heterodontiformes) is more closely related to lamniform than to carcharhiniform sharks (Martin and Palumbi 1993; Martin 1995). A molecular phylogeny of the prickly shark, *Echinorhinus cookei* (Echinorhinidae), based on the nuclear small ribosomal subunit (18S rRNA) gene combined with a fragment of the mitochondrial cytochrome b gene suggested that *E. cookei* is closely related to a sister group of Squalidae+Hexanchidae (Bernardi and

Powers 1992). Interrelationships of lamniform sharks were investigated with the cytochrome b and the NADH-2 gene (Naylor et al. 1997).

Sharks and rays are traditionally known as two separated orders of chondrichthyes fishes (Bigelow and Schroeder 1948, 1953). This view contrasts with the recent hypothesis (Compagno 1973, 1977, 1990) that sharks and rays should not be separated into two different groups at the level of order or higher taxa, but rather that Elasmobranchii were diversified into four monophyletic groups (Rajomorphii, including all rays, Squalomorphii, Squatinomorphii, and Galeomorphii). A molecular phylogenetic analysis based on the sequence of a 303-base pair region of the 12S rRNA gene of sharks from four different orders (Heterodontiformes, Lamniformes, Hexanchiformes, Squaliformes), a ray species (order Rajiformes), and a holocephalan species (order Chimaeriformes) provided evidence for the separation of sharks and rays (Dunn and Morrissey 1995). Another phylogeny based on the sequence of 732-base pair fragments of the mitochondrial cytochrome b gene also provided evidence for the dichotomous classification of sharks and rays into two orders (Selachii and Batoidei) within the superorder Squalea (Kitamura et al. 1996).

Kitamura et al. (1996) also identified a close relationship between the angel shark, *Squatina nebulosa*, and the saw shark, *Pristiophorus japonicus*, and that the dog fish, *Squalus japonicus*, was a sister species of a *Squatina-Pristiophorus* monophyletic group. Squatiformes, Squaliformes and Pristioriformes sharks are generally grouped as sibling taxa together with rays (Bigelow and Schroeder 1948, 1953; Compagno 1973, 1977).

In this chapter the evolutionary relationships of the three species of *Squatina* from southern Brazil, and between them and other groups of sharks, were investigated with the use of 401-base pair sequences of the cytochrome b gene.

4.2. Material and Methods

4.2.1. Samples

Tissue (muscle, liver or heart) samples from three individuals of each species of angel shark (*S. argentina*, *S. guggenheim* and *S. occulta*) from southern Brazil were collected by Clara Emilie Boeckmann, from the Department of Oceanography of the Fundação Universidade de Rio Grande, in Rio Grande do Sul State, Brazil. The samples were obtained from commercial fishing vessels, operating between latitudes 30°00'S and 34°30'S, from March 1994 to August 1995.

4.2.2. DNA extraction

DNA was extracted with the same procedure as in 2.2.2.

4.2.3. DNA amplification

PCR (polymerase chain reaction) was used to amplify 401-base pair sequences of the mitochondrial DNA cytochrome b gene from each species. The primers used were L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and H15149 (5'-GCCCCTCAGAATGATATTTGTCCTCA-3') of Irwin et al. (1991). Amplification reactions were performed according to 2.2.3.

4.2.4. Purification of PCR product

DNA was purified with the same procedure as in 2.2.4.

4.2.5. DNA Sequencing

The DNA sequencing procedure was identical to that described in 2.2.5.

4.2.6. Phylogenetic Analyses

The DNA sequences of the three species of *Squatina* (Squatiniformes) were analyzed together with those of twelve species of sharks: *Squalus acanthias* (Squaliformes), *Carcharhinus plumbleus*, *C. porosus*, *Sphyna lewini*, *S. tiburo*, *Prionace glauca*, *Negaprion brevirostris*, *Galeocerdo cuvier* (Carcharhiniformes), *Carcharodon carcharias*, *Lamna nasus*, *Isurus oxyrynchus*, and *Isurus paucus* (Lamniformes). One species of ray (*Urolophus concentricus*) was used as the outgroup. Phylogenetic analyses were performed with the Phylogenetic Analysis Using Parsimony (PAUP) [version 4.0d61] program of Swofford (1997). Maximum parsimony trees were obtained with the heuristic search algorithm (tree-bisection-and-reconnection) with random addition and delayed-character-transformation optimization. Ratio of transversions (Tv) to transitions (Ti) of 1:1, 3:1 and 5:1 were used. Bootstrap analyses were performed by means of the heuristic search algorithm with 10 random taxon additions and the tree-bisection-and-reconnection option in each of 300 replicates. Neighbor-joining analysis (Saitou and Nei 1987) was also performed (bootstrap analysis with 1,000 replicates), using the maximum-likelihood

distance parameters. Sequence of *Urolophus concentricus* (GenBank accession number U27265) was from Martin (1995). Sequence of *Squalus acanthias* (Gen Bank accession number M91184) was from Bernardi and Powers (1992) with exception of the first 98 nucleotides positions. which were reconstructed based on the sequences of the other fifteen species (nucleotide positions that were constant across all species were retained and variable nucleotides were coded according to the ambiguity code of the International Union of Biochemists). The other eleven sharks sequences (Gen Bank accession numbers L08031 to L08043) were from Martin and Palumbi (1993).

4.3. RESULTS

Within the 401-bp fragment of the cytochrome b gene. 21 variable nucleotide sites were identified among the three species of angel sharks (Figure 4.2). Of these. seventeen (81.0%) were pyrimidine transitions (eleven at third positions and six at a first positions). three (14.3%) were purine transitions (two at third positions and one at first position). and one (4.7%) was a transversion (at the third position). Only one of the observed substitutions would result in an amino acid substitution: the purine transition at nucleotide 70 would result in the exchange of alanine for threonine. The sequences of *S. guggenheim* and *S. occulta* differed at five nucleotide sites while these two together differed from *S. argentina* in sixteen sites.

Among the 180 variable nucleotides in the 401-bp sequence data set of 15 shark and a ray species. 153 parsimony-informative characters were identified. Because transitions accumulate faster than transversions in animal mitochondrial

DNA, different ratios of Tv:Ti were used in the MP analyses (Tv:Ti=1:1, 3:1, and 5:1). MP analyses that considered transversions only or were highly weighted for transversions (Tv:Ti=10:1, for example) were not performed in this study because only one transversion was identified among the *Squatina* sequences.

A sister relationship between *S. guggenheim* and *S. occulta* was identified in both MP (Figure 4.3) and NJ analyses (Figure 4.4). Bootstrap values that supported this monophyletic group were 95, 98, and 90 (for MP analysis with Tv:Ti=1:1, 3:1, and 5:1), and 93 (NJ analysis). Both MP and NJ analyses supported a monophyletic group comprising the three species of *Squatina* with bootstrap values of 100 (Figures 4.3 and 4.4).

In both MP and NJ analyses, the dogfish shark, *S. acanthias*, was observed to be the species more closely related to the three species of *Squatina* from southern Brazil.

Each MP analysis yielded only one shortest tree. MP analyses where transversions were weighted five and three times more than transitions generated trees with length of 1,207 and 846, respectively (CI = 0.510; RI = 0.630 for Tv:Ti=5:1, and CI = 0.465; RI = 0.506 for Tv:Ti=3:1). The tree for the MP analysis where transitions and transversions were equally weighted had a length of 563 (CI = 0.469; RI = 0.514). Figure 4.3 shows the maximum parsimony tree obtained from the analysis where Tv:Ti=3.1. The NJ tree obtained using matrices calculated with maximum-likelihood method (δ=0.213; Tv:Ti=5.098) is shown in Figure 4.4.

Figure 4.2. Variation in DNA sequence among the three species of angel sharks (*Squatina argentina, S. guggenheim, and S. occulta*) in a 401-bp region of the cytochrome b mitochondrial gene. Dots represent nucleotides that are identical to the *S. argentina* sequence. The top line gives the inferred amino acid sequence according to the single letter code of the International Union of Biochemists. Numbers at the end of the first and second line indicate the position numbers in the protein and nucleotide sequences, respectively.

```
                M   T   T   N   I   R   K   T   H   P   10
S.argentina     atg acc act aat atc cga aaa acc cat cca   30
S.guggenheim    ... ... ... ... ... ... ... ... ... ...
S.occulta       ... ... ... ... ... ... ... ... ... ...

                L   F   K   I   I   N   H   T   L   I   20
S.argentina     ctt ttt aaa att att aac cac acc tta att   60
S.guggenheim    ... ... ... ... ... ..t ... ... c.. ...
S.occulta       ... ... ... ... ... ..t ... ... ... ...

                D   L   P   A   P   S   N   I   S   I   30
S.argentina     gat cta cca acg cct tcc aac att tca atc   90
S.guggenheim    ... t.. ... g.. ... ... ... ... ... ...
S.occulta       ... t.. ... g.. ... ... ... ... ... ...

                W   W   N   F   G   S   L   L   G   L   40
S.argentina     tga tga aac ttt ggt tca ctt tta gga ctt  120
S.guggenheim    ... ... ... ... ... ... ... ... ... ...
S.occulta       ... ... ... ... ... ... ... ... ... ...

                C   L   I   I   Q   I   L   T   G   L   50
S.argentina     tgc ctt att atc caa atc cta aca ggc cta  150
S.guggenheim    ... ... ... ... ... ... ... ... ... ...
S.occulta       ... ... ... ... ... ... ... ... ... ...

                F   L   A   M   H   Y   T   A   D   I   60
S.argentina     ttt tta gcc ata cat tac act gca gac att  180
S.guggenheim    ..c ... ... ... ... ... ... ... ... ...
S.occulta       ..c ... ... ... ... ... ... ... ... ...

                S   L   A   F   S   S   V   I   H   I   70
S.argentina     tcc ctt gcc ttc tcc tca gta att cac att  210
S.guggenheim    ... ... ... ... ... ... ... ... ... ...
S.occulta       ..a ... ... ... ... ... ... ... ... ...

                C   R   D   V   N   Y   G   W   L   I   80
S.argentina     tgc cga gat gtt aat tac gga tga cta atc  240
S.guggenheim    ... ... ... ... ... ... ... ... ... ...
S.occulta       ... ... ... ... ... ..t ... ... ... ...

                R   N   I   H   A   N   G   A   S   I   90
S.argentina     cgt aat atc cat gct aat gga gca tca att  270
S.guggenheim    ... ... ..t ... ... ... ... ... ... ...
S.occulta       ... ... ..t ... ... ... ... ... ... ...

                F   F   I   C   I   Y   L   H   I   A  100
S.argentina     ttt ttt atc tgt atc tac tta cat att gcc  300
S.guggenheim    ... ... ..t ... ..t ... c.g ..c ... ...
S.occulta       ... ... ..t ... ..t ... c.g ..c ... ...
```

```
                R   G   L   Y   Y   G   S   Y   L   S   110
S.argentina     cga gga tta tat tat ggc tcc tac ctt tcc 330
S.guggenheim    ... ... c.. ... ... ... ... ... ..c ...
S.occulta       ... ... c.. ... ... ... ... ... ... ...

                K   E   T   W   N   I   G   V   I   L   120
S.argentina     aaa gaa aca tga aat atc gga gta att tta 360
S.guggenheim    ... ... ... ... ... ..t ... ... ... ..g
S.occulta       ... ... ... ... ... ..t ... ... ... ...

                L   F   L   L   M   A   T   A   F   V   130
S.argentina     tta ttc cta tta ata gca aca gcc ttt gta 390
S.guggenheim    ... ..t t.. c.. ... ... ... ... ... ...
S.occulta       ... ..t t.. c.. ... ... ... ... ... ...

                V   M   Y                               133
S.argentina     ggc tat gta tt                          401
S.guggenheim    ..t ... ... ..
S.occulta       ..t ... ... ..
```

Figure 4.3. Maximum parsimony tree (heuristic search, 300 replicates) based on 401-bp sequences of the cytochrome b mitochondrial DNA gene of fifteen species of sharks and one species of ray (*U. concentricus*), which was used as outgroup. The top values correspond to nucleotides differences between branchs. The bottom values correspond to a MP bootstrap analysis where transitions were weighted three times more than transversions.

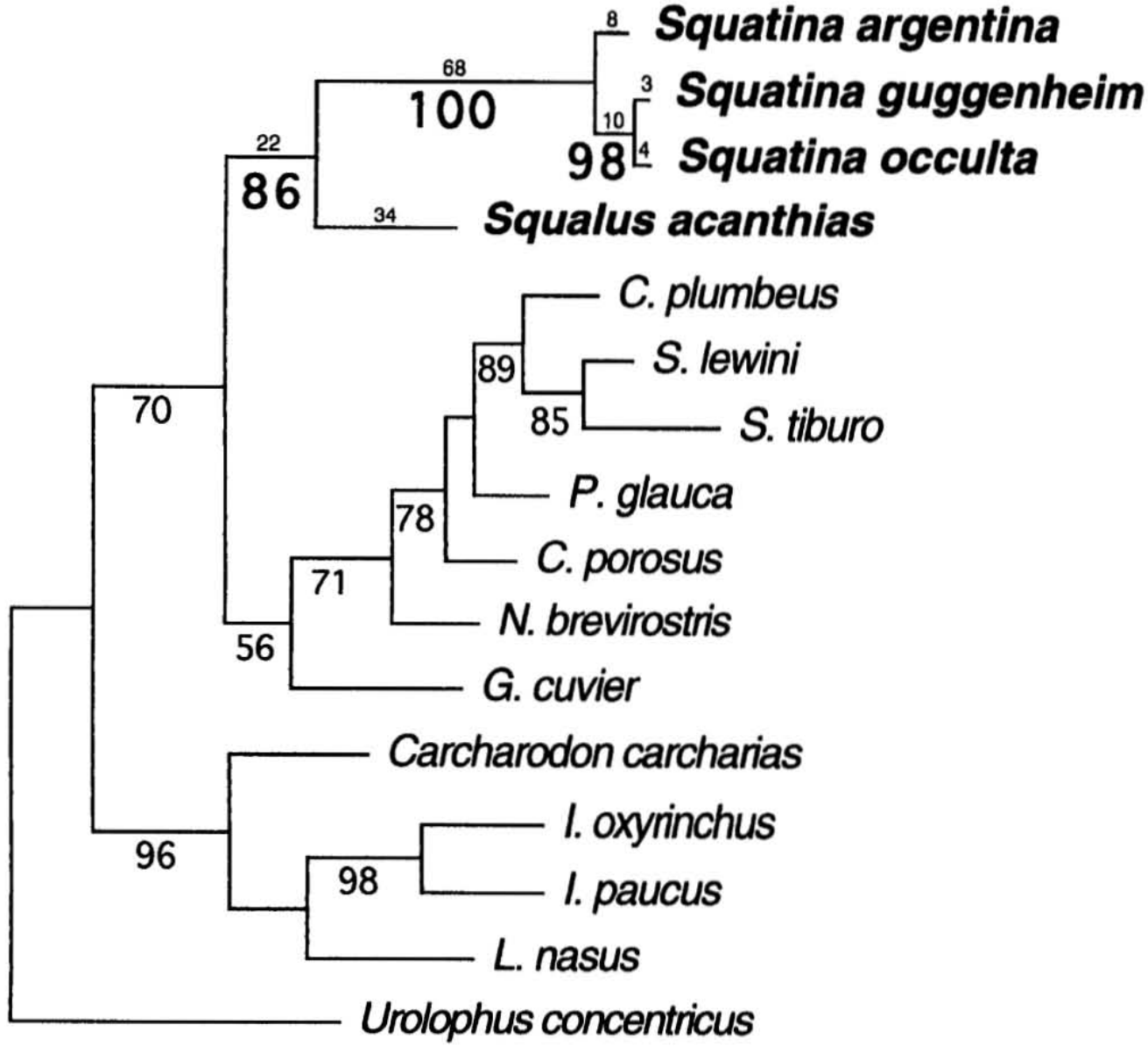
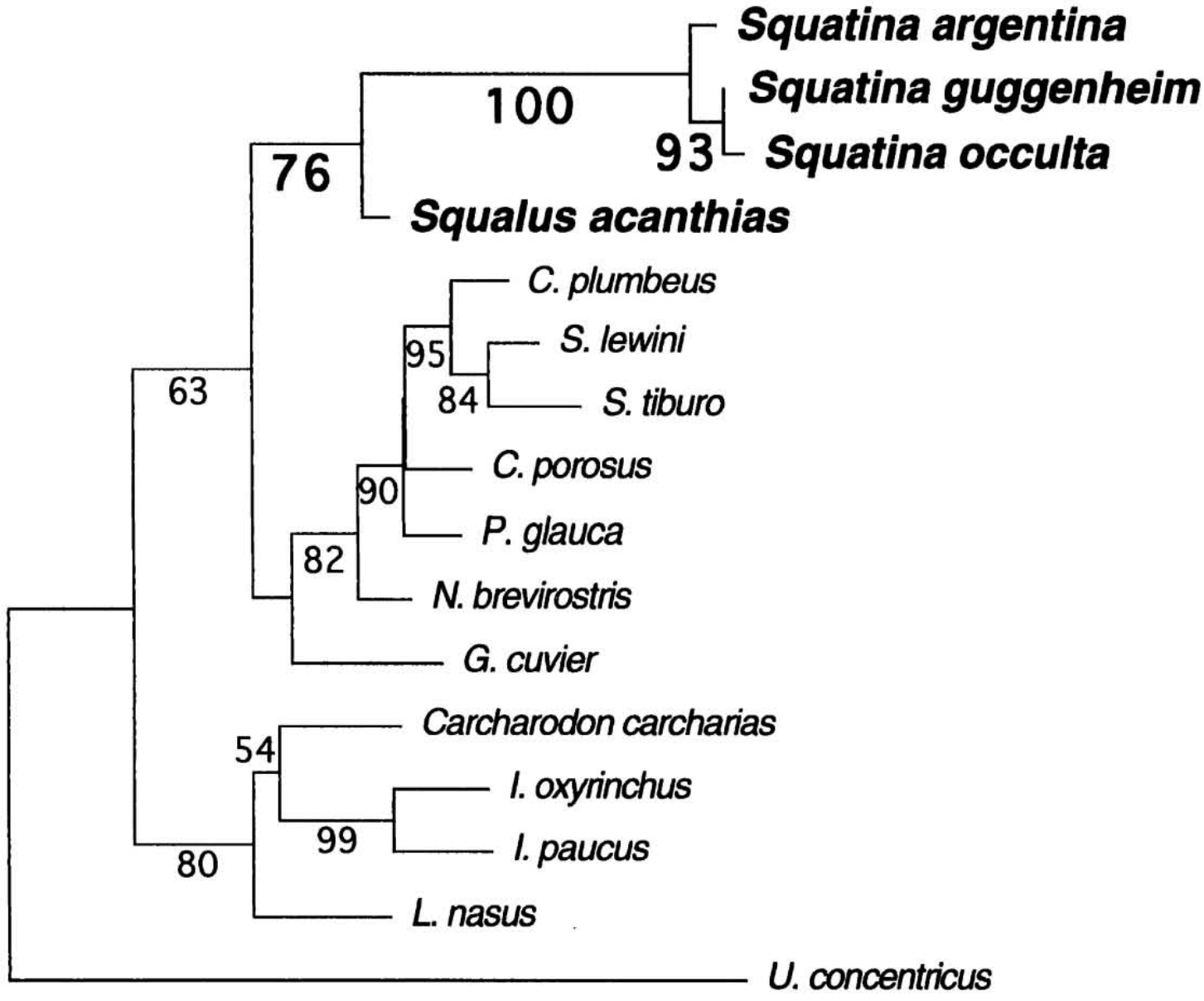

Squatina argentina
Squatina guggenheim
Squatina occulta
Squalus acanthias
C. plumbeus
S. lewini
S. tiburo
P. glauca
C. porosus
N. brevirostris
G. cuvier
Carcharodon carcharias
I. oxyrinchus
I. paucus
L. nasus
Urolophus concentricus
8
68
100
3
10
98
4
22
86
34
89
85
70
78
71
56
96
98

Figure 4.4. Neighbor joining bootstrap tree (1,000 replicates) using maximum-likelihood distance (Tv:Ti=5.078; δ=0.213), inferred from 401-bp sequences of the cytochrome b mitochondrial DNA gene of fifteen species of sharks and one species of ray (*U. concentricus*), which was used as outgroup. Numbers indicate bootstrap values.


Squatina argentina
Squatina guggenheim
100
93
Squatina occulta
76
Squalus acanthias
C. plumbeus
S. lewini
95
63
84
S. tiburo
C. porosus
90
P. glauca
82
N. brevirostris
G. cuvier
Carcharodon carcharias
54
I. oxyrinchus
99
I. paucus
80
L. nasus
U. concentricus

4.4. DISCUSSION

All the phylogenetic analyses performed in this study indicate that the *Squatina* species from southern Brazil constitute a monophyletic group, with *Squalus acanthias* as the species more closely related to them among the species examined in this study.

The newly described species *S. occulta* is more closely related to *S. guggenheim* than to *S. argentina* in all the MP (Figure 4.3) and NJ (Figure 4.4) analyses, although *S. occulta* and *S. guggenheim* differ in morphological aspects (e.g. presence or absence of dorsal spines, tooth formula), in parameters of growth (maximum total length, maximum total weight, total length and total weight at birth), and in parameters of reproduction (fecundity, total length at sexual maturity, diameter and mass of the mature follicle) (Vooren and Silva 1991). Individuals of *S. occulta* are larger and heavier than *S. guggenheim* at all stages of life, and they also have higher fecundity than the latter.

The molecular phylogeny obtained here suggests that the similarities between *S. occulta* and *S. argentina* are shared ancestral characteristics. *S. occulta* and *S. argentina* have many similarities in parameters of growth and reproduction. Furthermore, the spatial distributions of these two species overlap partially in the continental shelf of southern Brazil in depths of 60 to 200 meters, whereas *S. guggenheim* rarely is found in depths greater than 80 meters (Vooren and Silva 1991).

Previous biochemical studies, based on isozyme analysis and isoelectric focusing of sarcoplasmic proteins, identified three genetic distinct morphotypes of the angel sharks from southern Brazil (Solé-Cava and Levy 1987). Morphotypes I and II of *Squatina* spp. were more closely related to each other than either was to morphotype III. However, the authors did not identify the species represented by each morphotype. Later, morphotypes I and II were identified as *S. guggenheim* and *S. occulta*, respectively, by Vooren and Silva (1991). The results obtained here based on cytochrome b sequences support the suggestion of a sister relationship between morphotypes I and II of *Squatina* spp. (Solé-Cava and Levy 1987).

The close relationship between *S. occulta* and *S. guggenheim* observed in this molecular study is supported by a reproductive characteristic, the number of functional ovaries. The present phylogeny suggests that a single functional ovary is a synapomorphy between *S. occulta* and *S. guggenheim*. *S. argentina* is the only of the three angel sharks species from southern Brazil that maintains the "ancient character" (Vooren and Silva 1991) of paired functional ovaries in the female, while *S. occulta* and *S. guggenheim* have a single functional ovary on the left-hand side of the body cavity. Other species of *Squatina*, such as *S. japonica*, *S. dumeril*, and most specimens observed of *S. californica*, possess only the left ovary functional, whereas *S. oculata* and *S. squatina* have two functional ovaries (Dodd et al. 1983; Natanson and Cailliet 1986).

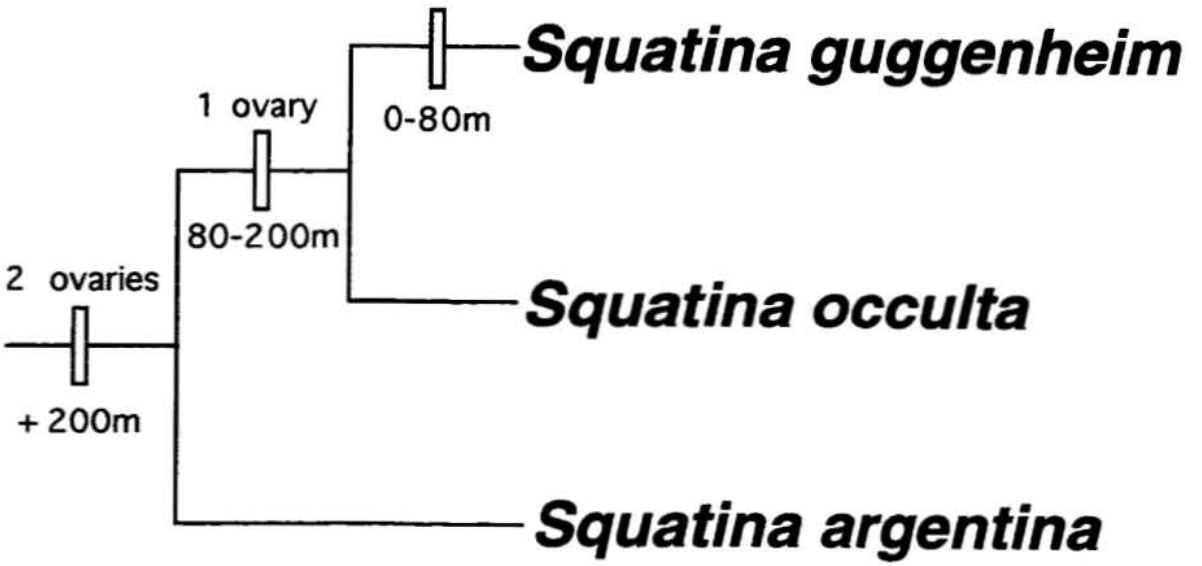
Ovaries are paired structures in most elasmobranchs, but it was observed that they can be asymmetrical in adult sharks of the orders Carcharhiniformes,

Pristiophoriformes and Squatiniformes (Wourms 1977; Natason and Cailliet 1986; Wourms et al., 1993). Phylogenetic analyses of mitochondrial DNA sequences had shown that the angel sharks (Squatiniformes) and saw sharks (Pristiophoriformes) are sister groups (Kitamura et al. 1996).

The evolution of species with a single functional ovary from the ancestral condition with two functional ovaries seems to have occurred in at least two independent evolutionary events, since the phylogenetic analyses performed here showed that Squatiniformes and Carcharhiniformes are genetically separated by another group of sharks (Squaliformes) that present two functional ovaries.

Vooren and Silva (1991) suggested that speciation in angel sharks may occur with minor changes in body form, and major changes in parameters of growth and reproduction. The results of the present study suggested that *S. occulta* and *S. guggenheim*, the two species that share the reproductive characteristic of a single functional ovary, are the most closely related pair of species among the three species from southern Brazil. *S. argentina*, which has two functional ovaries and has the highest fecundity (range of 7 to 11 embryos), is the only species that lives in depths lower than 200 meters (up to 500m). *S. occulta* is found in depths between 60 and 200m and has an intermediate fecundity between the three species (4 to 10 embryos), while *S. guggenheim* occurs from zero to 60m and has the lowest fecundity (3 to 8 embryos) (Vooren and Silva 1991; Silva 1996).

Figure 4.5. Hypothesis of evolution of three species of angel sharks (*S. argentina, S. guggenheim, and S. occulta*) from southern Brazil as predicted by mitochondrial cytochrome b gene sequences.


Squatina guggenheim
1 ovary
0-80m
80-200m
2 ovaries
Squatina occulta
+ 200m
Squatina argentina

The present phylogenetic analysis suggests that evolution of the genus *Squatina* in southeastern South America waters occurred from deeper to shallower waters (Figure 4.5). The results indicate that *S. argentina* was the first species of *Squatina* to occupy the continental shelf in depths of 200m or more. Fossil records suggest that the genus *Squatina* has existed since the Upper Jurassic (Capetta 1987). *S. occulta* and *S. guggenheim* have evolved more recently and speciation probably occurred as an adaptation to life in shallower waters on different types of sea bottom. The different color patterns observed in the three species have also been cited as evidence of adaptation to different types of ocean bottom (Vooren and Silva 1991). If this suggestion is true, *S. guggenheim*, the species that lives from 0 to 80m, is the most recent species among the three *Squatina* from southern Brazil.

4.5. CONCLUSIONS

The phylogenetic analyses performed in this study indicates that the three species of the genus *Squatina* from continental shelf off southern Brazil constitute a monophyletic group, and that the most parsimonious interrelationship between these species is: (*S.argentina* (*S. occulta*, *S. guggenheim*)). All the analyses suggest that the recently described species *S. occulta* is more closely related to *S. guggenheim* than to *S. argentina*. The results of this study suggest that evolution of the genus *Squatina* in southeastern South American waters occurred from deeper to shallower waters and from an ancestral condition of two functional ovaries to a derived condition of a single functional ovary, observed in the sister species *S. occulta* and *S. guggenheim*.

CHAPTER 5

GENETIC ANALYSIS OF THE STOCK STRUCTURE OF THE RED SNAPPER, *Lutjanus purpureus*, IN NORTHERN BRAZIL

5.1. Introduction

The Caribbean red snapper (*Lutjanus purpureus*, Poey 1867) is one of 65 species of the genus *Lutjanus* (Bloch 1790), which inhabits the coral reefs and rocky areas in tropical and subtropical waters throughout the world (Allen 1985). This species is found only in the tropical western Atlantic Ocean, on fishing grounds of the continental shelf and oceanic banks in Central and South America from Honduras to northeastern Brazil, and in the Caribbean Sea (Carpenter and Nelson 1971; Allen 1985).

The fishery for red snapper is the second most important in the north and northeastern Brazilian coastal waters, after the spiny lobster fishery (Ivo and Hanson 1982). In Brazilian waters, *L. purpureus* has been commercially exploited since 1961 with a maximum total annual catch of 7.547 metric tons in 1977, and an average annual catch of 5.937 metric tons between 1967 and 1987. Such catch levels are close to the maximum sustainable yield, estimated at 6.310 metric tons/year (Ivo and Sousa 1988). After 1987 there was a quick decline in the snapper fishery until 1991 when the annual catch reached a minimum of 1.200 metric tons. Since 1992, a slow recovery of the fisheries has been recorded (Salles 1997).

According to Allen (1985), spawning of *L. purpureus* occurs mainly during spring and summer, but Ivo and Hanson (1982) found major concentrations of spawning females in northern and northeastern Brazilian waters in both March/April and October. This could occur either because the species consists of one stock with two annual spawning periods, with each mature female spawning twice a year, or alternatively, because it consists of two stocks defined through differences in breeding time, with each group of females spawning once a year.

Based on reproduction, feeding habits and growth, Ivo and Hanson (1982) hypothesized that the red snapper population was segregated in two stock units, separated by the discharge of the Amazon River as an environmental barrier along the 47°W meridian. Two sub-areas, Sa-I (43°-46°W) and Sa-II (47°-49°W), with oceanographic and environmental differences were identified, each stock occupying one sub-area. Sa-I has an average salinity of 36‰ and water temperature of 28°C, whereas Sa-II is characterized by very low salinity of 20‰, because of the influence of the freshwater from the Amazon River, with temperature of 27°C (Ivo and Hanson 1982). Recently, statistical differences were found to occur in five of eight morphometric relationships and one meristic relationship between individuals from Sa-I and Sa-II (Salles 1997) in support of Ivo and Hanson's hypothesis (Ivo and Hanson 1982).

Restriction endonuclease analysis of mitochondrial cytochrome b and 12S ribosomal RNA gene fragments have been used as a simple method for species and stock identification of 13 species of western Atlantic snappers (Chow et al. 1993).

The phylogenetic relationships of 14 species of snappers (10 from the genus *Lutjanus*) occurring in the western Atlantic Ocean were studied by Sarver et al. (1996) with DNA sequences from portions of two mitochondrial genes, 12S rRNA and cytochrome b. Sequence variation in *L. purpureus* was not examined by any of these studies.

In the present chapter the occurrence of one or more stocks of *L. purpureus* in northern and northeastern Brazil and the phylogenetic placement of this species among the western Atlantic snappers was investigated with the use of 307-bp sequences of the mitochondrial cytochrome b gene.

5.2. Material and Methods

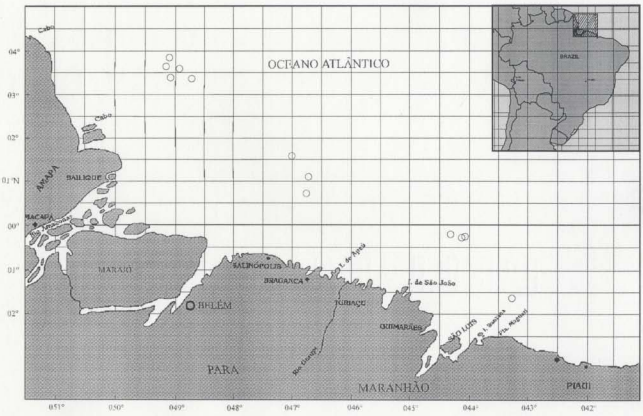
5.2.1. Samples

Tissue (muscle or heart) samples were obtained of twelve red snappers (*L. purpureus*) from northern Brazil caught between latitudes 03°50'N - 01°54'S, and longitudes 49°16'W - 42°53'W (Figure 5.1). All samples were collected by Mr. Rodrigo de Salles, from the Laboratório de Ciências do Mar of the Universidade Federal do Ceará, Brazil.

5.2.2. DNA extraction

DNA was extracted with the same procedure as in 2.2.2.

Figure 5.1. Map of northern Brazil showing the twelve locations where samples of red snapper (*Lutjanus purpureus*) were collected. The geographic coordinates for each sample are shown on Table 5.1.


OCEANO ATLANTICO
BRAZIL
AMAPÁ
MARAJÓ
PARÁ
MARANHÃO
PIAUI
BELÉM
SALINÓPOLIS
BRAGANÇA
TURIAÇU
OLINDA
SÃO LUIS
Cabo
BAILIQUE
MACAPÁ
Rio Amazonas
Rio Gurupi
Pta. Mangues
04°
03°
02°
01°N
00°
01°
02°
051°
050°
049°
048°
047°
046°
045°
044°
043°
042°

5.2.3. DNA amplification

PCR (polymerase chain reaction) was used to amplify 307-base pair sequences of the mitochondrial DNA cytochrome b gene from the collected samples. The primers used were L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and CYTBH (5'-GGCAAATAGGAATTATCATTC-3') (Irwin et al. 1991; Palumbi 1996). Amplification reactions were performed according to 2.2.3.

5.2.4. Purification of PCR product

DNA was purified with the same procedure as in 2.2.4.

5.2.5. DNA Sequencing

The DNA sequencing procedure was identical to that described in 2.2.5.

5.2.6. Genetic Heterogeneity and Phylogenetic Analyses

Genetic heterogeneity among samples were tested with the Monte Carlo χ^2 test of Roff and Betzen (1989) from REAP (Restriction Enzyme Analysis Package); 5000 resamplings of the data matrix were used. Phylogenetic analyses were performed with the PAUP [version 4.0d61] program of Swofford (1997). Maximum parsimony trees were identified with the heuristic search algorithm (tree-bisection-and-reconnection) with random addition and delayed-character-transformation optimization. Tranversions and transitions were weighted 3:1. Bootstrap analyses were performed by means of the heuristic search algorithm with 10 random taxon additions and the tree-bisection-and-reconnection option in each of 300 replicates.

5.3. RESULTS

Within the 307-bp amplified segment, five variable sites were identified among the twelve individual snapper. All substitutions occured in the third codon position and would not result in an amino acid substitution. These variable sites define four genotypes that differ by one to three nucleotide substitutions (Figure 5.2). A maximum parsimony network identified two different monophyletic groups: one including genotypes A and B, and the other including genotypes C and D with bootstrap value of 100% (Figure 5.3).

Genotype A was found in five individuals collected northwestern of the Amazon River mouth (samples LP01-LP05 in Table 5.1) and in one individual collected in front of the river mouth (sample LP07). Genotype B was detected in two samples collected just in front of the river mouth (LP06 and LP08). Genotype C was from three samples collected southeastern of the Amazon River mouth (LP09-LP11), and genotype D was represented by a single individual (LP12) from a southeastern location off Maranhao State (Table 5.1; Figure 5.1).

The Monte Carlo χ^2 test indicates significant differences of genotype distributions between the samples from northwest and southeast of the Amazon River mouth ($\chi^2 = 12$, $df = 3$, $p < 0.05$).

Bootstrap value of 99 for the *L. purpureus* genotypes were found when they were analyzed along with ten other species of the genus *Lutjanus* from Sarver et al. (1996). *L. campechanus* was the species most closely related to *L. purpureus* in the analysis (Figure 5.4).

Figure 5.2. Variation in *L. purpureus* DNA sequences within a 307-bp region of the mitochondrial cytochrome b gene. Dots represent nucleotides that are identical to genotype A. The top line gives the inferred amino acid sequence according to the single letter code specified by the International Union of Biochemists. Numbers at the end of the first and second line indicate the position numbers in the protein and nucleotide sequences, respectively.

```
            M   N   Q   S   T   K   N   H   P   L   L   K   12
Genotype A atg aat cag tct acg aaa aac cac cca tta cta aaa  36
Genotype B ... ... ... ... ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ... ... ... ...
Genotype D ... ... ... ... ... ... ... ... ... ... ... ...

            I   A   N   D   A   S   S   D   L   P   A   P   24
Genotype A att gct aac gac gcg tct agt gat ctc ccc gca ccc  72
Genotype B ... ... ... ... ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ... ... ... ...
Genotype D ... ... ... ... ... ... ... ... ... ... ... ...

            S   N   I   S   V   W   W   N   F   G   S   L   36
Genotype A tcc aat att tca gta tga tga aac ttt ggc tcc cta  108
Genotype B ... ... ... ... ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ... ... ... ...
Genotype D ... ... ..c ... ... ... ... ... ... ... ... ...

            L   G   L   C   L   I   A   Q   I   L   T   G   48
Genotype A ctt ggc ctt tgc tta att gcc caa att cta aca gga  144
Genotype B ... ... ... ..t ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ..c ... ... ...
Genotype D ... ... ... ... ... ... ... ... ..c ... ... ...

            L   F   L   A   M   H   Y   T   S   D   I   T   60
Genotype A ctt ttc ctc gcc ata cat tac acc tcc gac atc aca  180
Genotype B ... ... ... ... ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ... ... ... ...
Genotype D ... ... ... ... ... ... ... ... ... ... ... ...

            M   A   F   S   S   V   A   H   I   C   R   D   72
Genotype A ata gcc ttc tca tca gtc gcc cac atc tgc cga gat  216
Genotype B ... ..t ... ... ... ... ... ... ... ... ... ...
Genotype C ... ..t ... ... ... ... ... ... ... ... ... ...
Genotype D ... ..t ... ... ... ... ... ... ... ... ... ...

            V   N   Y   G   W   L   I   R   N   L   H   A   84
Genotype A gta aat tac gga tgg cta atc cgc aac ctc cat gcc  252
Genotype B ... ... ... ... ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ... ... ..c ...
Genotype D ... ... ... ... ... ... ... ... ... ... ..c ...

            N   G   A   S   F   F   F   I   C   I   Y   L   96
Genotype A aat ggt gcc tcc ttc ttc ttc atc tgc atc tac ctc  288
Genotype B ... ... ... ... ... ... ... ... ... ... ... ...
Genotype C ... ... ... ... ... ... ... ... ... ... ... ...
Genotype D ... ... ... ... ... ... ... ... ... ... ... ...

            H   I   G   R   G   L                           102
Genotype A cac atc ggc cga ggt ctt t                       307
Genotype B ... ... ... ... ... ... .
Genotype C ... ... ... ... ... ... .
Genotype D ... ... ... ... ... ... .
```

Table 5.1. Date of collection, location, and genotypes of samples of red snappers (*Lutjanus purpureus*) used in this study

Sample Code	Date of collection	Location	Genotype
LP01	29 Mar 96	03°28'N; 49°03'W	A
LP02	29 Mar 96	03°40'N; 49°16'W	A
LP03	27 Mar 96	03°50'N; 49°09'W	A
LP04	26 Mar 96	03°38'N; 48°55'W	A
LP05	25 Mar 96	03°26'N; 48°43'W	A
LP06	12 Mar 96	01°34'N; 47°01'W	B
LP07	10 Mar 96	01°02'N; 46°40'W	A
LP08	10 Mar 96	00°42'N; 46°44'W	B
LP09	04 Feb 96	00°14'S; 44°32'W	C
LP10	03 Feb 96	00°13'S; 44°12'W	C
LP11	03 Feb 96	00°13'S; 44°12'W	C
LP12	26 Jan 96	01°40'S; 43°19'W	D

Figure 5.3. Maximum parsimony network based on 307-bp sequences of the cytochrome b mitochondrial DNA gene of 4 different genotypes (A,B,C,D) of *Lutjanus purpureus*. Numbers show nucleotide differences between branches. Bootstrap value is 100%.

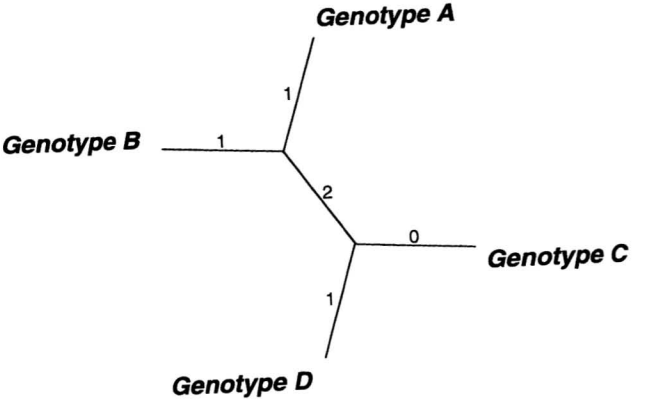
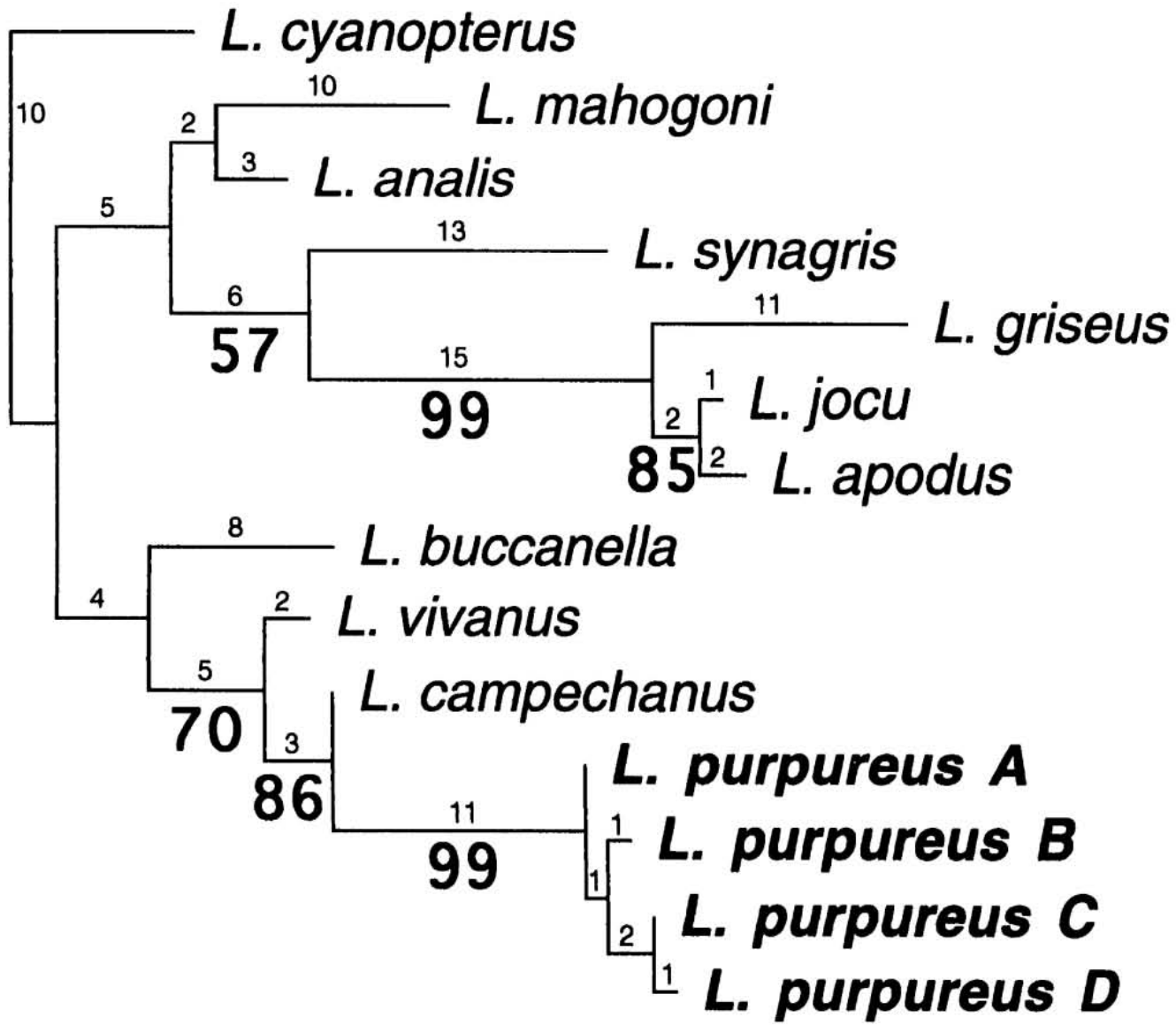

Genotype A
1
Genotype B
1
2
0
Genotype C
1
Genotype D

Figure 5.4. Maximum parsimony tree based on 229-bp sequences of the cytochrome b mitochondrial DNA gene of eleven species of red snapper (genus *Lutjanus*), including 4 different genotypes (A,B,C,D) of *Lutjanus purpureus*. Sequences of the other *Lutjanus* species (*L. vivanus, L. cyanopterus, L. buccanella, L. mahogoni, L. analis, L. synagris, L. griseus, L. jocu, L. apodus,* and *L. campechanus*) are from Sarver et al. (1996). Top numbers are the number of nucleotides differences between branches. Bottom numbers are bootstrap values.


L. cyanopterus
L. mahogoni
L. analis
L. synagris
L. griseus
L. jocu
L. apodus
L. buccanella
L. vivanus
L. campechanus
L. purpureus A
L. purpureus B
L. purpureus C
L. purpureus D
10
10
2
3
5
13
6
57
11
15
99
1
2
85
2
8
4
2
5
70
3
86
11
99
1
1
2
1

5.4. DISCUSSION

The identification of four different genotypes among only twelve specimens of *L. purpureus* sampled off northern Brazil indicates that this species has high genetic diversity in the studied area. The clade consisting of two genotypes (A. B) northwest and two genotypes (C. D) southeast of the discharge of the Amazon River mouth (along 47°W) supports a recent morphological study. which suggested that the *L. purpureus* population on the continental shelf of northern Brazil comprises two stock units occupying relatively segregated territories (Salles. 1997). These results also agree with an early study based on reproduction. growth and feeding habits of this species in northern Brazil (Ivo and Hanson. 1982).

Biological features of the two stock units of *L. purpureus* off northern Brazil have been characterized by Salles (1997). The first stock unit. occurring east of 47°W. consists of snappers with a lower growth rate and larger maximum and mean lengths than those found west of the Amazon River discharge. The second stock unit. occurring west of 47°W. consists of snappers with a higher growth rate and with smaller maximum and mean length than those found eastern of 47°W (Salles, 1997).

The maximum parsimony analysis with *L. cyanopterus*. the less closely related species to *L. purpureus* among the *Lutjanus* species from western Atlantic (Sarger et al. 1996). as the outgroup. yielded three minimum length trees (one of them shown in Figure 5.4) that suggest that genotypes A and B are the basal genotypes in the studied area. These where probably the first new genotypes to occur in snappers that occupied the South American continental shelf from northwest to southeast. migrating from the

Caribbean Sea. This assumption is supported by the endemic distribution of *L. campechanus*, the closest species to *L. purpureus*, in the Gulf of Mexico and Atlantic coast of the U.S. (Allen 1985). *L. purpureus* was frequently confused with *L. campechanus* in the Caribbean Sea due to their similar coloration and body proportions (Rivas 1966, Vergara 1980).

The present phylogenetic analysis also suggests that genotypes C and D, which occupy waters with average temperature of 28°C and high salinity of 36‰, probably is derived from the basal genotypes A and B, which occupy waters with average temperature of 27°C and low salinity of 20‰ (Ivo and Hanson 1982; Salles, 1997).

5.5. CONCLUSIONS

Four different genotypes of *L. purpureus* are distributed off northern Brazil. The grouping of two genotypes northwestern and two southeastern of the discharge of the Amazon River mouth suggests that the *L. purpureus* population on the continental shelf of northern Brazil comprises two stock units occupying areas with different salinity and temperature conditions. Phylogenetic analysis suggests that Genotypes A and B were the basal genotypes and the first to occupy the studied area. A sister relationship between *L. purpureus* and *L. campechanus* was identified by maximum parsimony analysis in agreement with previous morphological studies.

CHAPTER 6

GENETIC ANALYSIS OF THE STOCK STRUCTURE OF THE YELLOWFIN TUNA, *Thunnus albacares*, IN THE SOUTHWEST EQUATORIAL ATLANTIC OCEAN

6.1. Introduction

The yellowfin tuna (*Thunnus albacares*, Bonnaterre 1788) is the most abundant species of tuna in the Equatorial Atlantic Ocean (Fonteneau 1991). Since the early seventies, it has been believed that there is a continuous distribution of *T. albacares* in the equatorial Atlantic Ocean associated with East-West seasonal migration. This hypothesis is based upon longline and purse seine fisheries CPUE (capture per unit of effort) data and was first developed by Honma and Hisada (1971) and further elaborated by Yanez and Barbieri (1980) and Fonteneau (1981).

However, this model of stock structure for yellowfin tuna was not accepted by the International Commission for Conservation of the Atlantic Tuna (ICCAT) in their reports. For practical reasons a "two stock" hypothesis has been used in most assessments (Fonteneau 1991). According to the ICCAT's hypothesis the two stocks are separated at 30°W. Furthermore, Mahon and Mahon (1987) suggested that two stocks existed in the western Atlantic based on morphometric parameters. These stocks would have distinct seasonality with one stock moving from the Caribbean Sea to the north coast of Brazil, and sometimes mixing with the other stock that comes from Africa.

Transatlantic recoveries of tagged adult yellowfin tuna (Bard and Scott 1991) support the hypothesis of a single stock (Honma and Hisada 1971; Yanez and Barbieri 1980; Fonteneau 1981). The ICCAT acknowledge that it would not be possible to accept the "two stock" hypothesis if Bard and Scott's (1991) results are true (Fonteneau 1991).

A number of molecular genetic studies have been carried out on the stock structure of tuna species. Similarities between mitochondrial DNA sequences of albacore tuna (*T. alalunga*) from the Atlantic and the Pacific were reported by Graves and Dizon (1989), who did not find any restriction endonuclease sites that could distinguish the two stocks. Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA of *T. albacares* from the Pacific Ocean showed no evidence of genetic variation among individuals from distant geographic locations in the Pacific ocean (Scoles and Graves 1993). However, allozyme and restriction enzyme analyses have suggested that stocks of of *T. albacares* are distinct in the Atlantic, Indian, and Pacific oceans (Ward et al. 1997).

Bartlett and Davidson (1991) observed interspecific variation in 307-base pair (bp) segments of the mitochondrial cytochrome b gene between four species of tuna caught off the east coast of Canada: *Thunnus thynnus* (bluefin tuna), *T. obesus* (bigeye), *T. albacares* (yellowfin) and *T. alalunga* (albacore). RFLP analysis of mitochondrial DNA has shown that the bluefin tuna (*T. thynnus orientalis*) from the northern Pacific Ocean shares a larger number of restriction fragment sites with the albacore than with its Atlantic correlate (*T. thynnus thynnus*) (Chow and Inoue 1993).

Block et al. (1993) presented the first molecular phylogeny for the suborder Scombroidei (mackerels, tunas, and billfishes) based on a 600-bp region of the cytochrome b gene. The monophyly of the genus *Thunnus*, including *T. albacares*, *T. maccoyii* and *T. thynnus*, *T. alalunga* and *T.obesus*, was supported by bootstrap results. A close relationship between *T. albacares* and a *T.maccoyii* + *T.thynnus* clade was detected in this study and confirmed by Finnerty and Block (1995). A second molecular phylogeny for tuna species of the genus *Thunnus* based on partial sequences of the cytochrome b (292-bp) and ATPase (400-bp) genes was presented by Chow and Kishino (1995). They identified a close relationship between *T. albacares* and two others species of tuna (*T. alltlanticus* and *T. tonggol*) not studied by Block et al. (1993) or Finnerty and Block (1995).

Preliminary studies in northeastern Brazil (02°36'S–04°15'S and 32°34'W–33°45'W), where tuna fisheries is a growing industry, have shown differences in four of 19 morphometric characters between the Brazilian and the African yellowfin tunas (Neiva 1992).

In this chapter, partial nucleotide sequences (401-bp) of the cytochrome b gene were used to investigate genetic variation in the *T. albacares* stock from the southwest equatorial Atlantic Ocean off northeastern Brazil, and the placement of the Brazilian yellowfin tuna among the species of the genus *Thunnus*.

6.2. Material and Methods

6.2.1. Samples

Tissue (muscle, liver or heart) samples of 35 yellowfin tuna were collected in 11 voyages of the Research Vessel (RV) "Riobaldo" from March 1993 to November 1995, in the area between latitudes 01°00'N and 09°00'S and longitudes 29°00'W and 40°00'W in Brazilian waters (Figure 6.1). All samples were provided by Dr. Vera Lucia Vieira, from the Department of Fisheries Engineering of the Universidade Federal Rural de Pernambuco, Brazil.

6.2.2. DNA extraction

DNA was extracted with the same procedure as in 2.2.2.

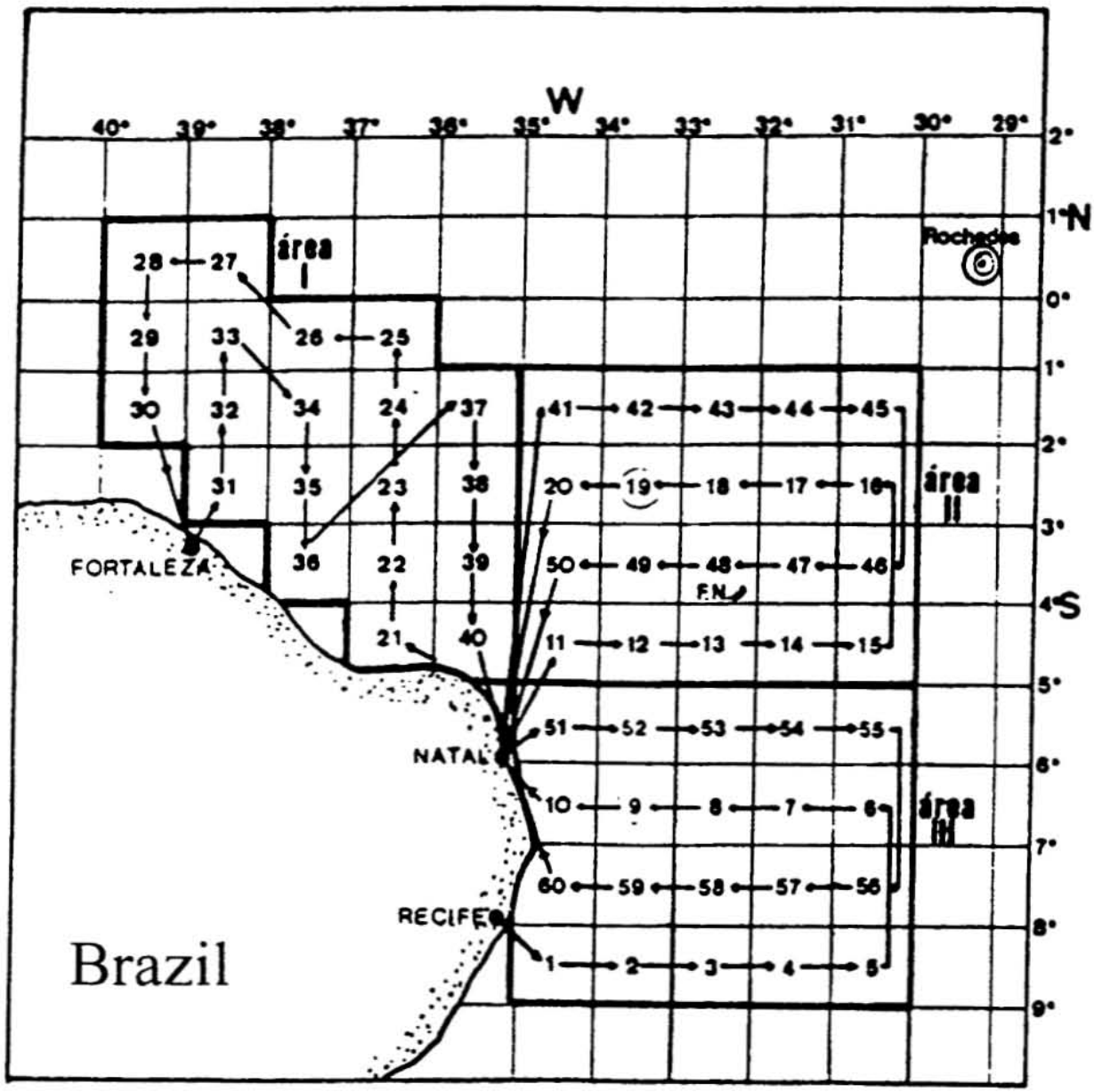
6.2.3. DNA amplification

PCR (polymerase chain reaction) was used to amplify 401-base pair sequences of the mitochondrial DNA cytochrome b gene from the collected samples. The primers used were L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and H15149 (5'-GCCCCTCAGAATGATATTTGTCCTCA-3') (Irwin et al. 1991). Amplification reactions were performed according to 2.2.3.

6.2.4. Purification of PCR product

DNA was purified with the same procedure as in 2.2.4.

Figure 6.1. Map of northern Brazil showing the areas (I-III) and sub-areas (1-60) in the southwest Equatorial Atlantic where samples of yellowfin tuna (*Thunnus albacares*) were collected, during the travels of the RV "Riobaldo". Routes of the cruises are shown by the lines in arrows. F.N. represents the Fernando de Noronha Islands.


W
40° 39° 38° 37° 36° 35° 34° 33° 32° 31° 30° 29°
2°
1°N
0°
1°
2°
3°
4°S
5°
6°
7°
8°
9°
área I
área II
área III
Rochedo
FORTALEZA
NATAL
RECIFE
F.N.
Brazil
28 27 26 25
29 33 34 24 37
30 32 35 23 38
31 36 22 39
21 40
41 42 43 44 45
20 19 18 17 16
50 49 48 47 46
11 12 13 14 15
51 52 53 54 55
10 9 8 7 6
60 59 58 57 56
1 2 3 4 5

6.2.5. DNA Sequencing

The DNA sequencing procedure was identical to that described in 2.2.5.

6.2.6. Phylogenetic Analyses

A consensus sequence of the genotypes of *T. albacares* identified by DNA sequencing was analyzed together with sequences of *T. albacares* from Canada (Bartlett and Davidson 1991) and the United States (Block et al. 1993). These sequences were analyzed alongside those of another six *Thunnus* species (*T. thynnus, T. alalunga, T. atlanticus, T. maccoyii, T. obesus, T. tonggol*) and three tuna species from other genera which were used as the outgroup (*Euthynnus affinis, Katsuwonus pelamis, Auxis thazard*). Phylogenetic analyses were performed with the PAUP [version 4.0d61] program of Swofford (1997). Maximum parsimony trees were identified with the heuristic search algorithm (tree-bisection-and-reconnection) with random addition and delayed-character-transformation optimization. Ratios of transversions to transitions of 10:1, 3:1, and 1:1 were used. Bootstrap analyses were performed by means of the heuristic search algorithm with 10 random taxon additions and the tree-bisection-and-reconnection option in each of 1,000 replicates. NJ analysis was performed using the Kimura-2-parameters (Saitou and Nei 1987). Sequences of *T. albacares* from Canada was from Bartlett and Davidson (1991). Sequences of *T. atlanticus* and *T. tongoll* (Gen Bank accession numbers D63492 and D63493) were from Chow and Kishino (1995). Sequences of *T. albacares* from U.S., *T. thynnus, T. alalunga, T. maccoyii, T. obesus* (L11556-L11560) and *E. affinis, K. pelamis, A. thazard* (L11534, L11539, and L11532, respectively) were from Block et al. (1993).

6.3. RESULTS

Within the 401-bp amplified segment of the cytochrome b gene, a single variable nucleotide site was identified among the 35 sampled individuals of *T. albacares* (Figure 6.2). It was a silent third position pyrimidine (C-T) transition at the 66th nucleotide position.

The most common genotype (T.albac-01), identified by a thymine at position 66, was found in 29 individuals, and the less common genotype (T.albac-02), identified by a cytosine at position 66, was observed in six individuals (Figure 6.2). Genotype T.albac-01 was detected in Areas I (sub-areas 33, 36, 37), II (sub-areas 19, 20), III (sub-areas 2, 4, 7, 8) and "Rochedos", while Genotype T.albac-02 was observed in Area II (sub-areas 19, 20), Area III (sub-area 9) and "Rochedos" (Figure 6.1). Three T.albac-02 were sampled in the "Rochedos" area and one specimen in each of the sub areas 09, 19, and 20.

The consensus sequence of the two genotypes differed in two positions from the 299-bp cytochrome b sequence of yellowfin tunas from the east coast of Canada (Bartlett and Davidson 1991). It also differs in three positions from the 286-bp fragment of the cytochrome b sequence of the U.S. east coast (Block et al. 1993). All substitutions are third position silent changes.

The three *T. albacares* sequences (from Brazil, Canada, and U.S.) were ranked in a monophyletic group in all the maximum parsimony analyses with bootstrap values (BV) of 59, 55, and 52, respectively for Tv:Ti ratios of 10:1, 3:1 and 1:1.

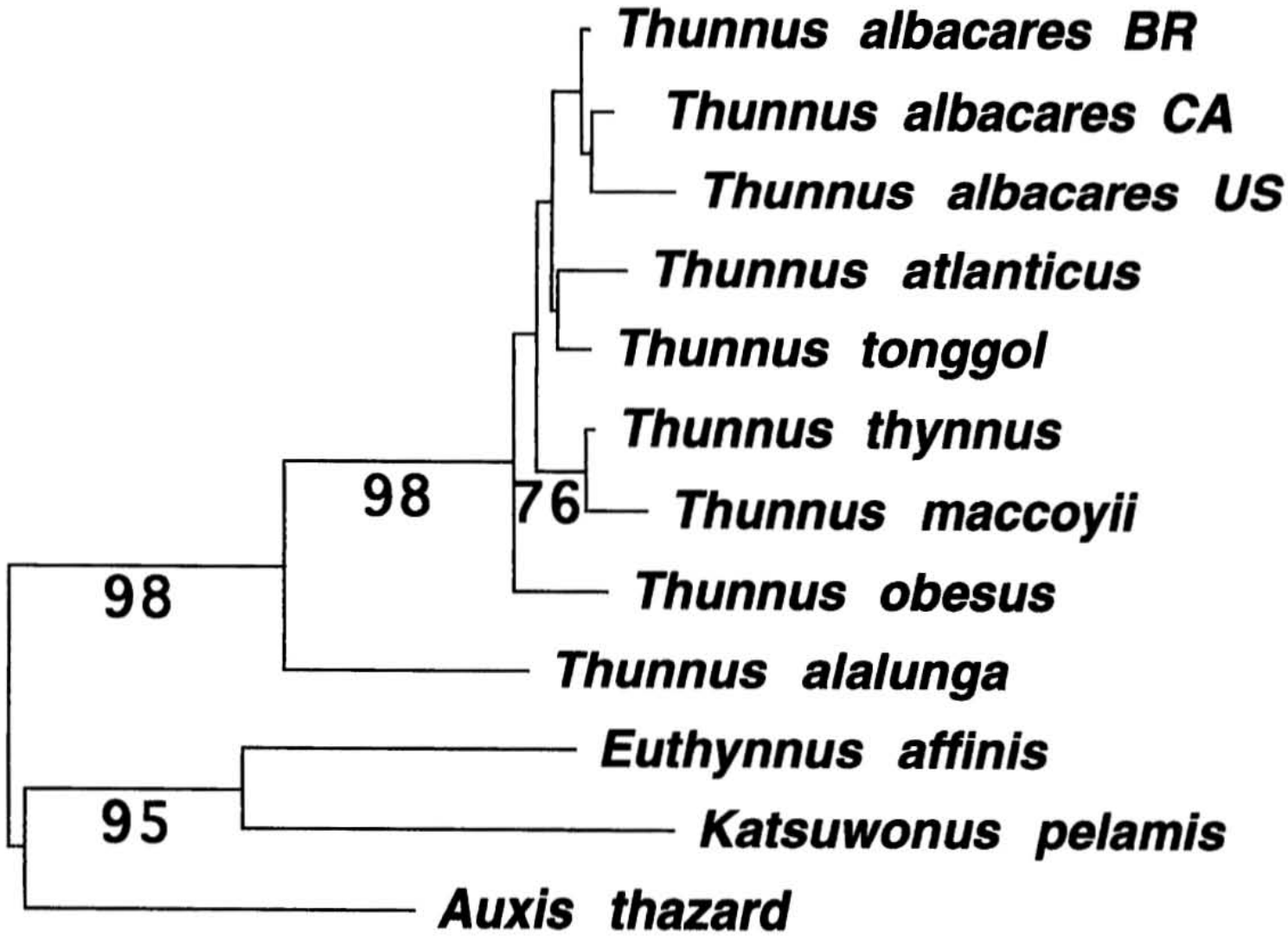
Another monophyletic group, constituted by *T. thynnus* and *T. maccoyii*, was observed (BVs=68, 64, 67) within a six-*Thunnus* clade (trees not shown).

Figure 6.3 shows the phylogenetic tree resulting from neighbor-joining analysis using distance matrices calculated by Kimura-2-parameters. The genetic distance between the three genotypes of *T. albacares* was 0.003. Yellowfin tuna from Canada and U.S. were more closely related to each other than to the yellowfin tuna from Brazil. The species more closely related to *T. albacares* were *T. atlanticus* and *T. tonggol*. The monophyly of the genus *Thunnus* was supported by BV of 98. *T. alalunga* was the only species not included in a monophyletic clade constituted by the other six species of the genus.

Figure 6.2. Variation in Brazilian *Thunnus albacares* mitochondrial DNA sequences within a 401-bp region of the cytochrome b mitochondrial gene. Dots represent nucleotides that are identical to genotype T.albac-01. The consensus sequence (*Thunnus albacares*-BR) used in the phylogenetic analysis has a total length of 286-base pairs and starts at the 133th position, and has the same length as the sequences obtained from GenBank. The top line gives the inferred amino acid sequence according to the single letter code specified by the International Union of Biochemists. Numbers at the end of the first and second line indicate the position numbers in the protein and nucleotide sequences, respectively.

```
             M   A   S   L   R   K   T   H   P   L   L   K   I   13
T.albac-01 atg gca agc ctc cga aaa act cac ccg cta cta aaa atc  39
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             A   N   D   A   L   V   D   L   P   T   P   S   N   26
T.albac-01 gct aac gac gca cta gtt gac ctt cct acc ccc tct aat  78
T.albac-02 ... ... ... ... ... ... ... ... ..c ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ..y ... ... ... ...

             I   S   A   W   W   N   F   G   S   L   L   G   L   39
T.albac-01 atc tct gca tga tga aac ttt ggc tca cta ctt ggc ctt 117
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             C   L   I   S   Q   I   L   T   G   L   F   L   A   52
T.albac-01 tgc ctt att tct caa atc ctt aca gga cta ttc ctc gca 156
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             M   H   Y   T   P   D   V   E   S   A   F   A   S   65
T.albac-01 ata cac tac acc cct gat gtc gaa tca gcc ttc gcc tca 195
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             V   A   H   I   C   R   D   V   N   F   G   W   L   78
T.albac-01 gta gcc cac att tgc cga gat gtc aac ttc ggt tga ctc 234
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             I   R   N   L   H   A   N   G   A   S   F   F   F   91
T.albac-01 atc cgg aac ctc cac gca aac ggg gcc tct ttc ttc ttt 273
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             I   C   I   Y   F   H   I   G   R   G   L   Y   Y   104
T.albac-01 atc tgc atc tac ttc cac atc ggc cga gga ctt tac tac 312
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             G   S   Y   L   Y   K   E   T   W   N   I   G   V   117
T.albac-01 ggc tct tac cta tac aag gaa aca tga aac atc gga gta 351
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             V   L   L   L   L   V   M   M   T   A   F   V   G   130
T.albac-01 gta ctc cta ctc cta gtt atg atg acc gcc ttc gtt ggc 390
T.albac-02 ... ... ... ... ... ... ... ... ... ... ... ... ...
T.albac-BR ... ... ... ... ... ... ... ... ... ... ... ... ...

             Y   V   L                                           133
T.albac-01 tac gtc ctt cc                                       401
T.albac-02 ... ... ... ..
T.albac-BR ... ... ... ..
```

Figure 6.3. Neighbor joining bootstrap tree (1,000 replicates) using Kimura-2-parameters, inferred from 286-bp mitochondrial cytochrome b sequences of 12 species of scombrids (GenBank accession numbers are given in 6.2.6). The three non-*Thunnus* species were used as outgroup. Numbers indicate bootstrap values.


Thunnus albacares BR
Thunnus albacares CA
Thunnus albacares US
Thunnus atlanticus
Thunnus tonggol
Thunnus thynnus
Thunnus maccoyii
Thunnus obesus
Thunnus alalunga
Euthynnus affinis
Katsuwonus pelamis
Auxis thazard
98
76
98
95

6.4. DISCUSSION

The level of nucleotide sequence divergence in yellowfin tuna from the northeastern coast of Brazil is very low. The single nucleotide difference in a 401-bp fragment of the cytochrome b gene between the genotypes T.albac-01 and T.albac-02 is not enough to determine the presence of two stocks in the studied area. Previous study on the Pacific yellowfin tuna also did not detect significant genetic differentiation among individuals from geographically distant locations, including samples from the Pacific and Atlantic oceans (Scoles and Graves 1993). The presence of different stocks or populations in determined area is generally associated with a reasonable amount of genetic variation (Scoles and Graves 1993; Avise 1994).

There is no indication that there are distinct stocks of *T. albacares* in the area studied. The genetic homogeneity of yellowfin tuna from the southwest equatorial Atlantic suggests that the stock present in that area shares a common gene pool. In two instances both genotypes were observed in the same sub-areas (19 and 20) of Area II (Figure 6.1), which indicates that tuna with different genotypes probably travel together in the same schools.

Comparison of mtDNA sequence data (Scoles and Graves 1993) with morphological data (Schaefer 1991, 1992) from the same locations in the Pacific Ocean have shown that although morphometric characters and gill-raker counts differed significantly, genetic differences were not observed. Scoles and Graves (1993) suggested that the morphological variation among Pacific yellowfin tunas was the consequence of the phenotypic plasticity of this species, evidencing that

morphological characters were environmentally influenced. Previous observation of greater morphological variation among yellowfin tuna from the Pacific than variation between the Atlantic and the Pacific tunas (Schaefer and Walford 1950) also supports this suggestion.

T. albacares is a migratory species (Collete and Naven 1993) and several studies of tagged adults have demonstrated that they do make trans-Atlantic crossings (Bard and Scott 1991) and can travel large distances between regions in the Pacific (Fink and Bayliff 1970; Bayliff 1984; Itano and Williams 1992). The circumtropical occurence of *T. albacares* larvae in both Atlantic and Pacific oceans (Nishikawa et al. 1985) suggests the existence of spawning areas throughout the tropical oceans and consequently allows gene flow between distant locations (Scoles and Graves 1993).

The results obtained in this study were in agreement with the hypothesis of gene flow in the Atlantic Ocean (Hornna and Hisada 1971), since different genotypes were observed in the same region. The neighbor joining results (Figure 6.3) show that the genotypes found in North Atlantic and South Atlantic are distinct.

The low frequency of occurrence of the genotype T.albac-02 is congruent with the concept that "unusual" mitochondrial DNA genotypes do not occur in high frequencies (Slatkin 1985). This condition has been observed in other fishes species, where high gene flow has been verified, such as marine catfishes of the family Ariidae (Avise et al. 1987), bluefish, *Pomatomus saltatrix* (Graves et al. 1992), and Greenland halibut, *Reinhardtius hippoglossoides* (Vis et al. 1997).

6.5. CONCLUSIONS

The low nucleotide variation in the mitochondrial DNA sequences of *T. albacares* from northeastern Brazil is consistent with the hypothesis that there is only a single stock of yellowfin tuna in the southwest Atlantic Ocean. The genetic homogeneity of the *T. albacares* stock occuring in the studied area suggests that the stock shares a common gene pool. It also implies that this species sustains sufficient gene flow in that area to prevent genetic variation. These results are similar to those obtained in the Pacific Ocean by Scoles and Graves (1993).

CHAPTER 7

GENERAL DISCUSSION

7.1. Evaluation of molecules as tools for systematic studies

The DNA sequences of mitochondrial genes are suitable for this study because of their recognized efficiency in resolving molecular phylogenetic relationships within and among species. Molecular data obtained in the different chapters of this thesis were also compared with previous morphological data because both views are informative and should be considered (Hillis et al. 1996). The results obtained in this thesis support the effectiveness of molecules in studying systematics and population genetics of marine vertebrates.

The use of the COI gene sequences alone, and in combination with other mitochondrial gene sequences, demonstrates that molecular phylogenies for cetaceans are sensitive both to the mitochondrial genes used and to the different methods of phylogenetic analyses.

The cytochome b gene has been shown to be a versatile molecular probe for investigating phylogenetic relationships among species, as in angel shark species from southern Brazil, and stock structure within species, as in the dolphin *S. fluviatilis*, yellowfin tuna (*T. albacares*) and red snapper (*L. purpureus*) from Brazilian waters. These results show that the cytochrome b gene is sufficiently variable for population studies and yet sufficiently conservative for phylogenetic studies (Meyer and Wilson 1990; Meyer, 1994).

7.2. Genetic variation in *Sotalia fluviatis* from Brazilian waters

This study has provided the first genetic evidence that the freshwater and marine ecotypes may be distinct. If this distinction is confirmed with a larger number of samples it will be an important guide to conservation actions for Brazilian environmental authorities, which should treat the two ecotypes as different populations. Although the identification of five different genotypes in the species along the marine coast indicated a the high genetic diversity, the occurrence of a common genotype in all six coastal locations suggests that there is sufficient gene flow to prevent significant genetic differentiation in the marine environment.

7.3. Molecular phylogeny of cetaceans and the placement of Physeteridae within Cetacea

The present study used the largest nucleotide data set ever assembled to examine phylogenetic relationships among cetaceans, combining DNA sequences of four mitochondrial genes. The results obtained in the present thesis show that the use of larger sequence data sets of few taxa for construction of trees and the use of fewer nucleotides per species and more species representing more taxa of the groups studied can yield different phylogenies (cf. Lecointre et al. 1993). Because the impact of species sampling may be stronger than the impact of sequence variation, the use of fewer nucleotides per species and more species representing more taxa of the groups

studied was recommended by Lecointre et al. (1993). If this assumption is correct the phylogenetic analyses of the COI sequence data may be the most accurate.

One of the observations of the present study was that neighbor joining analyses using maximum-likelihood parameters yielded essentially the same results as maximum-likelihood analyses alone. Although, the likelihood algorithm is considered to be superior to parsimony and neighbor joining methods as a means for estimating the 'correct phylogeny' when rates of evolution differ among lineages (Felsenstein 1981), maximum-likelihood is very computer time-intensive. If a neighbor joining analysis with maximum-likelihood parameters provides "full-blown" maximum-likelihood analyses in far less time, than neighbor joining may be legitimate to use instead the longer timing method.

The results in this thesis are not in agreement with the hypothesis of Milinkovitch et al. (1993, 1994) and Milinkovitch (1995) that baleen whales and sperm whales should be included in a common clade. The results obtained by Milinkovitch et al. (1993, 1994) were controversial at first because they suggested a rejection of the traditional view of toothed-whale monophyly. However, this interpretation has not been substantially supported by subsequent morphological (Heyning 1997) and paleontological evidence (Thewissen and Hussain 1993; Thewissen et al. 1994). Besides the present investigation, other molecular studies, such as those of Arnason and Gullberg (1994, 1996) have produced analyses contrary to Milinkovitch's hypothesis.

7.4. The identification of phylogenetic relationships of angel sharks from southern Brazil

The identification of relationships among the three species of angel sharks from southeastern South America is important as part of the ecological background for management of angel shark fisheries in Brazil. Among the three species, *S. argentina* is the one that has shown the lower number of individuals along the coastal waters of Rio Grande do Sul (Boeckmann 1996). The molecular evidence that these species constitute a single clade with a common ancestor, and that *S. argentina* is the oldest species, is important to the correct understanding of the phylogenetic relationships among these species of economical importance. This was the first study of molecular systematics of Brazilian shark species.

7.5. Identification of stock structures of red snapper and yellowfin tuna in Brazilian waters and its importance to management of fisheries

This study was the first that investigated the population structure of these two important commercial species. The observed differences in genetic patterns between *T. albacares* and *L. purpureus* may be attributed to their different life styles, and the characteristics of the areas where these species are distributed in Brazilian waters. Red snapper are distinct inhabitants of coral reef and rocky areas and do not exhibit long distance migrations (Allen 1985). The area where this species was sampled is highly influenced by the freshwater discharge of the Amazon River and has different

sub-areas with different salinity and temperature (Ivo and Hanson 1982). The isolation of stocks in different sub-areas is likely responsible for the low level of gene flow and allows genetic variation that was detected by the presence of different genotypes identified in the present study. Fisheries statistics and management should be based on the existence of the two stocks based on the results obtained in this study, as well as findings of Ivo and Hanson (1982) and Salles (1997). Yellowfin tuna is a pelagic migratory species able to make trans-Atlantic crossings (Bard and Scott 1991) and travel large distances between regions in the Pacific (Fink and Bayliff 1970; Bayliff 1984; Itano and Williams 1992). This characteristic allows a sufficient amount of gene flow and prevents genetic variation. The genetic identification of a single stock of yellowfin tuna is important for future regulation of this growing fisheries off northeastern Brazil.

7.6. Future of molecular systematics and population genetics studies of marine vertebrates in Brazil

The work described in this thesis was the first real attempt to study molecular systematics and population genetics of marine vertebrates from Brazil using DNA technology. Early studies of Brazilian species used allozymes and protein electrophoresis to study the systematics of fish species (e.g. Solé-Cava et al. 1983 and Solé-Cava and Levy 1987), but molecular techniques had never been used before in studies of marine mammals occurring in Brazilian waters or in studies of fish species from the north and northern regions of the Brazilian coast.

Future studies on molecular systematics of other marine vertebrates from Brazil, such as manatees (order Sirenia) and seals (order Pinnipedia), are necessary to clarify the phylogenetic relationships among species of these groups. Phylogenetic relationships of chondrichthyes and osteichthyes fishes economically exploited in Brazil should also be investigated. Other species of economical importance in Brazilian fisheries, such as sardines (Clupeidae), should be studied in relation to population genetics to guide fisheries management. Population genetic studies on the three species used in this thesis (*S. fluviatilis*, *T. albacares*, and *L. purpureus*) should be improved with the inclusion of a larger number of samples and the DNA sequences of other genes.

8. REFERENCES

Adachi, J., and Hasegawa, M. 1995. Phylogeny of whales: dependence of the inference on species sampling. Mol. Biol. Evol. 12: 177-179.

Adkins, R. M., Honeycutt, R. L., and Disotell, T. R. 1996. Evolution of the eutherian cytochrome c oxidase subunit II: heterogeneous rates of protein evolution and altered interaction with cytochrome c. Mol. Biol. Evol. 13 (10): 1393-1404.

Allard, M. W., Miyamoto, M. M., Jarecki, L., Kraus, F., and Tennant, M. R. 1992. DNA systematics and evolution of the artiodactyl family Bovidae. Proc. Natl. Acad. Sci. USA 89: 3972-3976.

Allen, G. R. 1985. FAO Species catalogue. Vol. 6. Snappers of the world: an annotated and illustrated catalogue of lutjanid species known to date. FAO Fish. Synop. 125(6): 1-208.

Alves-Jr., T. T. Ávila, F. J. C., Oliveira, J. A., Furtado-Neto, M. A. A. & Monteiro-Neto, C. 1996. Registros de cetáceos para o litoral do Estado do Ceará, Brasil. Arq. Ciên. Mar 30: 79-92.

Anderson, S., de Bruijn, M. H., Coulson, A. R., Eperon, I. C., Sanger, F., and Young, I. G. 1982. Complete sequence of bovine mitochondrial DNA conserved features of the mammalian mitochondrial genome J. Mol. Biol. 156 (4): 683-717.

Arnason, U. 1972. The role of chromosomal rearrangement in mammalian speciation with special reference to Cetacea and Pinnipedia. Hereditas 70: 113-118.

Arnason, U. 1974. Comparative chromosome studies in Cetacea. Hereditas 77: 1-36.

Arnason, U. 1982. Karyotype stability in marine mammals. Cytogenet. Cell. Genet. 33: 274-276.

Arnason, U., and Gullberg, A. 1994. Relationship of baleen whales established by cytochrome b gene sequence comparison. Nature 367: 726-728.

Arnason, U., and Gullberg, A. 1996. Cytochrome b nucleotide sequences and the identification of five primary lineages of extant cetaceans. Mol. Biol. Evol. 13(2): 407-417.

Arnason, U., Gullberg, A., and Widegren, B. 1991. The complete nucleotide sequence of the fin whale, *Balaenoptera physalus*. J. Mol. Evol. 33: 556-568.

Arnason, U., Bodin, K., Gullberg, A., Ledje, C., and Mouchaty, S. 1995. A molecular view of pinniped relationships with a particular emphasis on the true seals. J. Mol. Evol. 40: 78-85.

Avise, J. C. 1994. Molecular markers, natural history and evolution. Chapman & Hall, New York, N.Y.

Avise, J. C., Reeb, C. A., and Saunders, N. C. 1987. Geographic population structure and species differences in mitochondrial DNA of mouthbrooding marine catfishes (Ariidae) and demersal spawning toadfishes (Batrachoididae). Evolution 41: 991-1002.

Ayala, F. J. 1995. Future of science in Latin America. Science 267: 827-828.

Baker, C. S., Cipriano, F., and Palumbi, S. R. 1996. Molecular genetic identification of whale and dolphin products from commercial markets in Korea and Japan. Mol. Ecol. 5: 671-685.

Baker. C. S.. Palumbi. S. R.. Lambertsen. R. H.. Weinrich. M. T.. Calambokidis. J.. and O'Brien. S. J. 1990. Influence of seasonal migration on geographic distribution of mitochondrial DNA haplotypes in humpback whales. Nature 344: 238-240.

Baker. C. S.. Slade. R. W.. Bannister. J. L.. Abernethy. R. B.. Weinrich. M. T.. Lien.. J.. Urban. J.. Corkeron. P.. Calmabokidis. J.. Vasquez. O.. and Palumbi. S. R. 1994. Hierarchial structure of mitochondrial DNA gene flow among humpback whales *Megaptera novaeangliae*. world-wide. Mol. Ecol. 3: 313-327.

Bard. F. X.. and Scott. E. L. 1991. Traversees de thons albacares marques de cotes americaines aux cotes africaines. Document realise dans la cadre du programme YYP. ICCAT. Paris. France.

Barnes. L. G. 1984. Search for the first whale. Oceans 17: 20-23.

Barnes. L. G.. and McLeod. S.A. 1984. In: Jones. M.L.. Swartz. S.L.. and Leatherwood. S. (eds.). The Gray Whale. Academic Press. New York. pp 3-32.

Barnes. L. G.. Domning. D. P.. and Ray. C. E. 1985. Status of studies on fossil marine mammals. Mar. Mammal Sci. 1(1): 15-53.

Barros. N. B. 1991. Recent cetacean records for southeastern Brazil. Mar. Mammal Sci. 7(2): 296-306.

Bartlett. S. E.. and Davidson. W. S. 1991. Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochome b genes. Can. J. Fish. Aquat. Sci. 48: 309-317.

Bayliff, W. H. 1984. Migrations of yellowfin and skipjack tuna released in the central portion of the eastern Pacific ocean, as determined by tagging experiments. IATTC Internal Rep. 18: 1-107.

Bernardi, G., and Powers, D. A. 1992. Molecular phylogeny of the prickly shark, *Echinorhinus cookei*, based on a nuclear (18S rRNA) and a mitochondrial (cytochrome b) gene. Mol. Phylogen. Evol. 1(2): 161-167.

Bessho, Y., Ohama, T., and Osawa, S. 1992. Planarian mitochondria. I. Heterogeneity of cytochrome c oxidase subunit I gene sequences in the freshwater planarian, *Dugesia japonica*. J. Mol. Evol. 34(4): 342-330.

Best, R. C., and Da Silva, V. M. F. 1984. Preliminary analysis of reproductive parameters of the boutu, *Inia geoffrensis*, and the tucuxi, *Sotalia fluviatilis*, in the Amazon River system. Reports of the International Whaling Commission, Special Issue 6: 361-369.

Bigelow, H. B., and Schroeder, W. C. 1948. Fishes of the Western North Atlantic. Part 1. Yale University, New Haven, CT.

Bigelow, H. B., and Schroeder, W. C. 1953. Fishes of the Western North Atlantic. Part 2. Yale University, New Haven, CT.

Block, B. A., Finnerty, J. R., Stewart, A. F. R., and Kidd, J. 1993. Evolution of endothermy in fish: mapping physiological traits on a molecular phylogeny. Science 260: 210-214.

Bloch, M. E. 1790. Naturgeschichte der ausländischen Fische. Part 4. J. Morino, Berlin, Germany.

Boeckmann. C. E. 1996. Dinâmica populacional e avaliação de estoques de cações-anjo. *Squatina guggenheim* Marini. 1936. e *S. occulta* Vooren e Silva. 1991. na plataforma continental do Sul do Brasil. Master thesis. Fundação Universidade de Rio Grande. Rio Grande do Sul. Brazil.

Bonnaterre. J. P. 1788. Tableu encyclopédique et methodique des trois regnes de la nature. Ichthyologie. Paris. France.

Borobia. M. 1989. The distribution and morphometrics of *Sotalia fluviatilis*. M. Sc. Thesis. McGill University. Montreal. PQ.

Borobia. M.. Siciliano. S.. Lodi. L.. and Hoek. W. 1991. Distribution of the South American dolphin *Sotalia fluviatilis*. Can. J. Zool. 69: 1025-1039.

Brennin. R.. Murray. B. W.. Friesen. M. K.. Maieres. L. D.. Clayton. J. W.. and White. B.N. 1997. Population genetic structure of beluga whales (*Delphinapterus leucas*): mitochondrial DNA sequence variation within and among North American populations. Can. J. Zoo. 75 (5): 795-802.

Brown. J. M.. Pellmyr. O.; Thompson. J. N.. and Harrison. R. G. 1994. Phylogeny of Greya (Lepidopters: Prodoxidae). based on nucleotide sequence variation in cytochrome oxidase I and II: congruence with morphological data. Mol. Biol. Evol. 11: 128-141.

Brown. W. M.. George. M.. and Wilson. A. C. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. 76: 1967-1971.

Buchanan. F. C.. Friesen. M. K.. Littlejohn. R. P.. and Clayton. J. W. 1996. Microsatellites from the beluga whale *Delphinapterus leucas*. Mol. Ecol. 5: 571-575.

Cabot. E. L. and Beckenbach. A. T. 1989. Simultaneous editing of multiple nucleic acid sequences with Esee. Comput. Appl. Bio Sci. 5: 233-234.

Cantatore. P.. Roberti. M.. Pesole. G.. Ludovico. A.. Milella. F.. Gadaleta. M. N.. and Saccone. C. 1994. Evolutionary analysis of cytochrome b sequences in some perciformes: evidence for a slower rate of evolution than in mammals. J. Mol. Evol. 39: 589-597.

Cao. Y.. Adachi. J.. Janke. A.. Pääbo. S.. and Hasegawa. M. 1994. Phylogenetic relationships among eutherian orders estimated from inferred sequences of mitochondrial proteins: instability of a tree based on a single gene. J. Mol. Evol. 39: 519-527.

Cappetta. H. 1987. Chondrichthyes. II. Mesozoic and Cenozoic Elasmobranchii. Vol. 3B: handbook of paleoichthyology. Gustav Fischer. Stuttgart. Germany.

Carpenter. J. S.. and Nelson. W. R. 1971. Fishery potential for snapper and grouper in the Caribbean area and the Guianas. FAO Fish. Rep.. Rome. 7(1-2): 21-26.

Carr. S. M. 1998. Molecular systematics of New- and Old-World deer: implications for taxonomy. biogeography. and the evolution of antlers. Mol. Phylogen. Evol.. (submitted).

Carr. S. M.. and Hicks. S. A. 1997. Are there two species of marten in North America? Genetics and evolutionary relationships within *Martes*. In: Proulx. G.,

Bryant, H. N., and Woodard, P. M. (eds.). Martes: taxonomy, ecology, techniques and management. Provincial Museum of Alberta, Edmonton, AB.

Carr, S. M., and Hughes, G. A. 1993. The direction of hybridization between species of North american deer (*Odocoileus*) as inferred from mitochondrial cytochrome b sequences. J. Mammal. 74: 331-342.

Carr, S. M., and Marshall, H. D. 1991a. Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome b gene of the Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. Can. J. Fish. Aquat. Sci. 48: 48-52.

Carr, S. M., and Marshall, H. D. 1991b. A direct approach to the measurement of genetic variation in fish populations: applications of the polymerase chain reaction to studies of Atlantic cod (*Gadus morhua*). J. Fish Biol. 39 (suppl.A): 101-107.

Carr, S. M., and Perry, E. A. 1997. Intra- and inter-familial systematic relationships of phocid seals as indicated by mitochondrial DNA sequences. Mar. Mam. Sci. (genetics special pub.): 283-296.

Carr, S. M., Wroblewski, J. S., Snellen, A. J., and Howse, K. A. 1995. Mitochondrial DNA sequence variation and genetic stock structure of Atlantic cod (*Gadus morhua*) from bay and offshore locations on the Newfoundland continental shelf. Mol. Ecol. 4: 79-88.

Chen, E.Y. 1994. The efficiency of automated DNA sequencing. In: Adams, M. D., Fields, C., and Venter, J. C. Automated DNA sequencing and analysis. Academic Press, London.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Chow, S., and Inoue, S. 1993. Intra- and interspecific restriction fragment length polymorphism in mitochondrial genes of *Thunnus* tuna species. Bull. Natl. Res. Inst. Far Seas Fish. 30: 207-225.

Chow, S., and Kishino, H. 1995. Phylogenetic relationships between tuna species of the genus *Thunnus* (Scombridae: teleostei): inconsistent implications from morphology, nuclear and mitochondrial genomes. J. Mol. Evol. 41: 741-748.

Chow, S., Clarke, M. E., and Walsh, P. J. 1993. PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification. Fish. Bull. 91: 619-627.

Churchill, G. A., Von Haeseler, A., and Navidi, W. C. 1992. Sample size for a phylogenetic inference. Mol. Biol. Evol. 9: 753-769.

Collette, B. B., and Nauen, C. E. 1983. FAO species catalogue. Vol 2. Scombrids of the world: an annotated and illustrated catalogue of tunas, mackerels, bonitos and related species known to date. FAO Fish. Synop. 125 (2): 1-137.

Compagno, L. J. V. 1973. Interrelationships of living elasmobranchs. In: Greenwood, P. H., Miles, R. S., and Patterson, C. (eds.), Interrelationships of fishes. Academic Press, London. pp 15-61.

Compagno, L. J. V. 1977. Phyletic relationships of living sharks and rays. Amer. Zool. 17: 303-322.

Compagno, L. J. V. 1984. FAO Species catalogue. Vol. 4. Sharks of the world: an annotated and illustrated catalogue of sharks species known to date. Part 1. Hexanchiformes to Lamniformes. FAO Fish. Synop. 125 (vol.4. pt.1): 1-249.

Compagno, L. J. V. 1990. The evolution and diversity of sharks. In: Gruber, S. H. (ed.). Discovering sharks. American Littoral Society, NJ. pp 15-22.

Cooper, C. E., Nicholls, P., and Freedman, J. A. 1991. Cytochrome c oxidase: structure, function and membrane topology of the polypeptide subunits. Biochem. Cell Biol. 69: 586-607.

Cousseau, M. B. 1973. Taxonomia y biologia del pez angel, *Squatina argentina* Marini (Pisces, Squatinidae). Physis (B. Aires) Secc. A, 32(84): 175-195.

Cracraft, J. and Mindell, D. P. 1989. The early history of modern birds: a comparison of molecular and morphological evidence. In: Fernholm, B., Bremer, K., and Jorwall, H. K. (eds.). The hierarchy of life. Elsevier, Amsterdam, The Netherlands. pp. 389-403.

Crutcher, D. C. 1996. Population structure of Atlantic cod (*Gadus morhua*) in the Northwest Atlantic as determined by mitochondrial DNA sequence. B.Sc. (Honours) Thesis, Memorial University of Newfoundland, St. John's, NF.

Cummings, M. P., Otto, S. P., and Wakeley, J. 1995. Sampling properties of DNA sequence data in phylogenetic analysis. Mol. Biol. Evol. 12(5): 814-822.

Czelusniak, J., Goodman, M., Koop, B. F., Tagle, D. A., Shoshani, J., Braunitzer, G., Kleinschmidt, T. K., DeJong, W. W., and Matsuda, G. 1990. In: Genoways, H.H. (ed.). Current mammalogy, vol. 2. Plenum, New York, NY.

Da Costa, L. N. 1995. Future of science in Latin America. Science 267: 827-828.

Da Silva, V. M. F. 1994. Aspects of the biology of the Amazonian dolphins genus *Inia* and *Sotalia fluviatilis*. Ph.D. dissertation. University of Cambridge, Cambridge, UK.

Da Silva, V. M. F., and Best, R. C. 1994. Tucuxi, *Sotalia fluviatilis* (Gervais) 1953. In: Ridgway, S. H., and Harrison, R. J. (eds.). Handbook of marine mammals. Volume 5. Academic Press, London, pp 43-69.

Da Silva, V. M. F., and Best, R. C. 1996. *Sotalia fluviatilis*. Mammalian species 527: 1-7.

De Salle, R., Freedman, T., Prager, E. M., and Wilson, A. C. 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J. Mol. Evol. 26: 157-164.

Dizon, A. E., Lockye, C., Perrin, W. F., Demaster, D. P., and Sisson, J. 1992. Rethinking the stock concept: a phylogeographic approach. Conserv. Biol. 6: 24-36.

Dodd, J. M., Dodd, M. H. I., and Duggan, R. T. 1983. Control of reproduction in elasmobranch fishes. In: Rankin, J. C., Pitcher, T. J., and Duggan, R. T. (eds.). Control processes in fish physiology. Wiley and Sons, Inc., New York, NY. pp. 221-229.

Donoghue, M. J., and Sanderson, M. J. 1992. The suitability of molecular and morphological evidence in reconstruction of plant phylogeny. In: Soltis, D. E.,

and Doyle. J. J. (eds.). Molecular systematics of plants. Chapman & Hall. New York. NY. pp. 340-368.

Douzery. E. 1993. Evolutionary relationships among Cetacea based on the sequence of the 12SrRNA gene: possible paraphyly of toothed whales (odontocetes) and long separate evolution of sperm whales (Physeteridae). C. R. Acad. Sci. Paris. Life Sciences 316: 1511-1518.

Dumeril. A. M. C. 1806. Zoologie analytique. ou méthode naturelle de classification des animaux. Zool. Anal.. Paris. 102: 1-344.

Dunn. K. A. and Morrissey. J. F. 1995. Molecular phylogeny of elasmobranchs. Copeia 1995(3): 526-531.

Erken. A.. Laver. P.. and Frankenhuis. M. 1994. Castration and sterilization of an adult male *Hippopotamus*. Verh. Ber. Erkrg. Zootiere 36: 41-43.

Fang. Q.. Blocker. H. D.. and Black. W. C. 1995. Cladistics analysis of neartics *Deltocephalus*-like *leafhoppers* (Homoptera: Cicadellidae) using morphological and molecular data. Ann. Entomol. Soc. America 88 (3): 316-323.

Felsenstein. J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17: 368-376.

Felsenstein. J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Felsenstein. J. 1993. PHYLIP. version 3.5. University of Washington. Seatle.

Figueiredo. J. L. 1977. Manual de peixes marinhos do Sudeste do Brasil: I.Introdução. cações. raias e quimeras. Museu de Zoologia. Universidade de São Paulo. Brazil.

Fink, B. D., and Bayliff, W. H. 1970. Migrations of yellowfin and skipjack tuna in the eastern Pacific Ocean as determined by tagging experiments, 1952-1964. Inter-Am. Trop. Tuna Comm. Bull. 15: 1-227.

Finnerty, J. R., and Block, B. A. 1995. Evolution of the cytochrome b in the Scombroidei (Teleiostei): molecular insights into billfish (Istiophoridae and Xiphiidae) relationships. Fish. Bull. 93: 78-96.

Fonteles-Filho, A. A. 1989. Recursos pesqueiros: biologia e dinâmica populacional. Imprensa Oficial do Ceará, Fortaleza, Brazil.

Fonteneau, A. 1981. Dynamique de la population d'albacore (*Thunnus albacares*) de l'ocean Atlantique. These Doctorat. Université Paris VI, France.

Fonteneau, A. 1991. Modeling a single Atlantic yellowfin stock with a mixing model. SCRS Report 30: 272-285.

Fordyce, R.E. 1980. Whale evolution and Oligocene southern ocean environment. Palaeogeogr. Palaeoclimatol. Palaeoecol. 31: 319-336.

Fordyce, R.E. 1992. In: Prothero, D. R. and Berggren, W. A. (eds.), Eocene-Oligocene climatic and biotic evolution. Princeton University Press, Princeton, NJ. pp 368-381.

Gaida, I. H. 1995. Evolutionary aspects of gene expression in the Pacific angel shark, *Squatina californica* (Squatiniformes: Squatinidae). Copeia 1995(3): 532-554.

Gatesy, J. 1997. More DNA support for a Cetacea/Hippopotamidae clade: the blood-clotting protein gene γ-fibrinogen. Mol. Biol. Evol. 14(5): 537-543.

Gatesy, J., Hayashi, C., Cronin, M., and Arctander, P. 1996. Evidence from milk casein genes that cetaceans are close relatives of hippopotamid artiodactyls. Mol. Biol. Evol. 13: 954-963.

Gervais, P. 1853. Remarques sur les mammifères marins qui frequentent les côtes de la France et plus particulièrement sur une nouvelle espèce de Dauphin propre à la Méditerranée. Bulletin de la Société Centrale d'Agriculture et des comices agricoles du départment de l'Herault (Montpellier) 40: 140-156.

Gingerich, P. D., Smith, B. H., and Simons, E. L. 1990. Hind limbs of Eocene Basilosaurus: evidence of feet in whales. Science 249: 154-157.

Gingerich, P. D., Raza, S. M., Arif, M., Anwar, M., and Zhou, X. 1994. New whale from the eocene of Pakistan and the origin of cetacean swimming. Nature 368: 844-847.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S. G. 1996. Life with 6000 genes. Science 274: 562-567.

Goodman, M., Romero-Herrera, A. E., Dene, H., Czelusniak, J., and Tashian, R. E. 1982. Amino acid sequence evidence on the phylogeny of primates and other eutherians. In: Goodman, M. (ed.). Macromolecular sequences in systematic and evolutionary biology. Plenum Press, New York, NY. pp. 115-191.

Graur, D., and Higgins, D. G. 1994. Molecular evidence for the inclusion of cetaceans within the order Artiodactyla. Mol. Biol. Evol. 11: 357-364.

Graves, J. E., and Dizon, A. E. 1989. Mitochondrial DNA sequence similarity of the Atlantic and Pacific albacore tuna (*Thunnus alalunga*). Can. J. Fish. Aquat. Sci. 46: 870-873.

Graves, J. E., McDowell, J. R., Beardsley, A. M., and Scoles, D. R. 1992. Stock structure of the bluefish *Pomatomus saltatrix* along the mid-Atlantic coast. Fish. Bull. 90: 469-475.

Hafner, M. S., Sudman, P. D., Villablanca, F. X., Spradling, T. A., Demastes, J. W., and Nadler, S. A. 1994. Disparate rates of molecular evolution in cospeciating hosts and parasites. Science 265: 1087-1090.

Hagelberg, E., and Clegg, J. B. 1991. Isolation and characterization of DNA from archaeological bone. Proc. Royal Soc. London. Series B. Biol. Sci. 244: 45-50.

Hagelberg, E., Thomas, M. G., Cook-Jr., C.; Sher, A. V.; Baryshnikov, G. F., and Lister, A. M. 1994. DNA from ancient mammoth bones. Nature 370: 333-334.

Handt, O., Höss, M., Krings, M., and Pääbo, S. 1994. Ancient DNA: methodological challenges. Experientia 50: 524-529.

Hasegawa, M., and Adachi, J. 1996. Phylogenetic position of cetaceans relative to Artiodactyls: reanalysis of mitochondrial and nuclear sequences. Mol. Biol. Evol. 13(5): 710-717.

Hasegawa, M., Kishino, H., and Yano, T. 1985. Dating of human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22: 160-174.

Hasegawa, M., Kishino, H., and Saitou, N. 1991. On the maximum likelihood method in molecular phylogenetics. J. Mol. Evol. 32: 443-445.

Hasegawa, M., Adachi, J., and Milinkovitch, M. C. 1997. Novel phylogeny of whales supported by total molecular evidence. J. Mol. Evol. 44 (Suppl 1): S117-S120.

Hatefi, Y. 1985. The mitochondrial electron transport and oxidative phosphorylation system. Annu. Rev. Biochem. 54: 1015-1069.

Hedges, S. B. 1994. Molecular evidence for the origins of birds. Proc. Natl. Acad. Sci. USA 91: 2621-2624.

Hennig, W. 1966. Phylogenetics systematics (translated by Davis, D.D. and Zangerl, R.). University of Illinois Press, Urbana, IL.

Heyning, J. E. 1989. Comparative facial anatomy of beaked whales (Ziphiidae) and a systematic revision among the families of extant Odontoceti. Contr. Sci. Nat. Hist. Mus. Los Angeles 405: 1-64.

Heyning, J. E. 1997. Sperm whale phylogeny revisited: analysis of the morphological evidence. Mar. Mam. Sci. 13(4): 596-613.

Heyning, J. E., and Mead, J. G. 1990. Evolution of the nasal anatomy of cetaceans. In: Thomas, J., and Kastelein, R. (eds.). Sensory abilities of cetaceans. Plenum Press, New York. pp. 67-79.

Hicks, S. A., and Carr, S. M. 1991. Genetic analysis of a threatened subspecies, the Newfoundland pine marten (*Martes americana atrata*). In: Willison, J. H. M., Bondrup-Nielsen, S., Drysdale, C., Herman, T. B., Munro, N. W. P., and Pollock, T. L. (eds.). Science and management in protected areas. Elsevier, Amsterdam, The Netherlands. pp. 287-290.

Hicks, S. A., and Carr, S. M. 1995. A molecular genetic perspective on the biogeographic origins of American pine marten (*Martes americana*). In: Bondrup-Nielsen, S., Willison, J. H. M., and Munro, N. W. P. (eds.). Science and management in protected areas. Elsevier, Amsterdam, The Netherlands. pp. 278-284.

Hillis, D. M. 1987. Molecular versus morphological approaches to systematics. Ecol. Annu. Rev. Syst. 18: 23-42.

Hillis, D. M., and Moritz, C. 1990. Molecular systematics. Sinauer, Sanderland, MA.

Hillis, D. M., Moritz, C., and Mable, B. K. 1996. Molecular systematics, 2nd edition. Sinauer, Sanderland, MA.

Hillis, D. M., Huelsenbeck, J. P., and Cunningham, C. W. 1994. Application and accuracy of molecular phylogenies. Science 264: 671-677.

Honeycutt, R. L., Nedbal, M. A., Adkins, R. M., and Janecek, L. L. 1995. Mammalian mitochondrial DNA evolution: a comparison of the cytochrome b and cytochrome c oxidase II genes. J. Mol. Evol. 40: 260-272.

Honma, M., and Hisada, K. 1971. Structure of yellowfin tuna population in the Atlantic ocean. Bull. Far Seas Fish. Res. Lab. 4: 93-124.

Höös, M. S., and Pääbo, S. 1993. DNA extraction from Pleistocene bones by a silica-based purification method. Nucleic Acids Res. 21: 3913-3914.

Huelsenbeck, J. P., and Hillis, D. M. 1993. Success of phylogenetic methods in the four-taxon case. Syst. Biol. 42: 247-264.

Hughes, G. A. 1990. A molecular genetic analysis of hybridization between two species of deer (*Odocoileus*) in Western Canada. M.Sc. Thesis, Memorial University of Newfoundland, St. John's, NF.

Hughes, G. A., and Carr, S. M. 1993. Reciprocal hybridization between white-tailed deer (*Odocoileus virginianus*) and mule deer (*O. hemionus*) in western Canada: evidence from serum albumin and mtDNA sequences. Can. J. Zoo. 71: 524-530.

Irwin, D. M., and Arnason, U. 1994. Cytochrome b gene of marine mammals: phylogeny and evolution. J. Mammal. Evol. 2: 37-55.

Irwin, D. M., Kocher, T. D., and Wilson, A. C. 1991. Evolution of the cytochrome b gene of mammals. J. Mol. Evol. 32: 128-144.

Itano, D. G., and Williams, P. G. 1992. Analysis of yellowfin tagging data and related information collected by the Skipjack Survey and Assessment Programme. SPC Tuna and Billfish assessment Programme (TBAP) Tech. Rep. 28: 1-39.

Ivo, C. T. C., and Hanson, A. J. 1982. Aspectos da biologia e dinâmica populacional do pargo, *Lutjanus purpureus* Poey, no Norte e Nordeste do Brasil. Arq. Ciên. Mar 22: 1-41.

Ivo, C. T. C., and Sousa, M. J. B. 1988. Sinopse de informações sobre o pargo, *Lutjanus purpureus* Poey (Pisces: Lutjanidae), no Norte e Nordeste do Brasil. Arq. Ciên. Mar 27: 57-67.

IUCN, The World Conservation Union. 1991. Dolphins, porpoises and whales of the world: the IUCN red data book (compiled by Klinowska, M.). Gland, Swizerland, and Cambridge, U.K.

Jefferson, T. A., Leatherwood, S., and Webber, M. A. 1993. FAO species identification guide: marine mammals of the world. FAO, Rome. pp. 1-320.

Kessing, B., Croom, H., Martin, A., McIntosh, C., McMillian, W. O., and Palumbi, S. 1989. The simple fool's guide to PCR. University of Hawaii, Honolulu, HI.

Kimura, T., Ozawa, T., and Pastene, L. A. 1997. Sample preparation and analysis of mitochondrial DNA from whale baleen plates. Mar. Mam. Sci. 13(3): 495-498.

Kitamura, T., Takemura, A., Watabe, S., Taniuchi, T., and Shimizu, M. 1996. Molecular phylogeny of the sharks and rays of superorder Squalea based on mitochondrial cytochrome b gene. Fisheries Science 62(3): 340-343.

Kivlichan, D. S. G. 1997. Biogeographic origins of gadig fishes as inferred from mitochondrial cytochrome oxidase I sequences. B.Sc. (Honours) Thesis, Memorial University of Newfoundland, St. John's, NF.

Klima, M. 1995. Cetacean phylogeny and systematics based on the morphogenesis of the nasal skull. Aquatic Mamm. 21: 70-89.

Klug, W. S., and Cummings, M. R. 1994. Concepts of Genetics, 4th edition. Macmillian College Pub., Cliffs, NJ.

Kraus, F., and Miyamoto, M. M. 1991. Syst. Zool. 40: 117-130.

Krettek, A., Gullberg, A., and Arnason, U. 1995. Sequence analysis of the complete mitochondrial DNA molecule of the hedgehog, *Erinaceus europaeus*, and the phylogenetic position of the Lipotyphla. J. Mol. Evol. 41: 952-957.

Kulu, D. D. 1972. Evolution and cytogenetics. In: Ridgway, S. H. (ed.), Mammals of the sea, biology and medicine. Charles C. Thomas, Springfield, IL. pp. 503-527.

Lander. E. S. 1996. The new genomics: global views of biology. Science 274: 536-539.

Lecointre. G.. Philippe. H.. Van Lê. H. L.. LeGuyader. C. 1993. Species sampling has a major impact on phylogenetic inference. Mol. Phylogen. Evol. 2(3): 205-224.

Ledje. C.. and Arnason. U. 1996. Phylogenetics analyses of complete cytochrome b genes of the order Carnivora with particular emphasis on the Caniformia. J. Mol. Evol. 42: 135-144.

LeDuc. R. G. 1997. A Systematic Study of the Delphinidae (Mammalia: Cetacea) using Cytochrome B Sequences. PhD Thesis. University of California. CA.

Lewin. B. 1997. Genes VI. Oxford University Press. Oxford. UK.

Ling. J. 1974. The integument of marine mammals. In: Harrison. R. (ed.). Functional anatomy of marine mammals. vol.2. Academic Press. London. UK. pp. 1-44.

Mahon. R.. and Mahon. S. 1987. Seasonality and migration of pelagic fishes in the eastern Carribean. FAO Fish. Rep. 393:1-270.

Maisey. J. G. 1984. Higher elasmobranch phylogeny and biostratigraphy. Zool. J. Linnean Soc. 82: 33-54.

Marini. T. L. 1930. Nueva especie de pez angel. *Rhina argentina* N. sp. Physis 10: 5-7.

Marini. T. L. 1936. Revision de las especies de la familia Squatinidae en las aguas Argentinas (*Squatina guggenhein* N. sp.). Physis 12: 19-30.

Martin. A. P. 1995. Mitochondrial DNA sequence evolution in sharks: rates, patterns, and phylogenetic inferences. Mol. Biol. Evol. 12(6): 1114-1123.

Martin, A. P., and Palumbi, S. R. 1993. Protein evolution in different cellular environments: cytochrome b in sharks and mammals. Mol. Biol. Evol. 10(4): 873-891.

Martin, A. P., Naylor, G. J. P., and Palumbi, S. R. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357: 153-155.

McElroy, D., Moran, P., Bermingham, E., and Kornfield, I. 1991. REAP: the Restriction Enzyme Analysis Package, version 4.0 edition. University of Maine, Orono.

McLeod, S. A., Whitmore, F. C., and Barnes, L. G. 1993. In: Burns, J. J., Montague, J. J. and Cowles, C. J. (eds.), The bowhead whale. Allen Press, Lawrence, KS. pp. 45-70.

Meyer, A. 1994. Shortcomings of the cytochrome b gene as a molecular marker. Trends Ecol. Evol. 9: 278-280.

Meyer, A., and Wilson, A. C. 1990. Origin of tetrapods inferred from their mitochondrial DNA affiliation to lungfish. J. Mol. Evol. 31: 359-364.

Milinkovitch, M. C. 1995. Molecular phylogeny of cetaceans prompts revision of morphological transformations. Trends Ecol. Evol. 10: 328-334.

Milinkovitch, M. C., Meyer, A., and Powell, J. R. 1994. Phylogeny of all major groups of cetaceans based on DNA sequences from three mitochondrial genes. Mol. Biol. Evol. 11(6): 939-948.

Milinkovitch, M. C., Orti, G., and Meyer, A. 1993. Revised phylogeny of whales suggested by mitochondrial ribosomal DNA sequences. Nature 361: 346-348.

Milinkovitch, M. C., Orti, G., and Meyer, A. 1995. Novel phylogeny of whales revisited but not revised. Mol. Biol. Evol. 12(3): 518-520.

Milinkovitch, M. C., LeDuc, R. G., Adachi, J., Fanir, F., Georges, M., and Hasegawa, M. 1996. Effects of character weighting and species sampling on phylogeny reconstruction: a case study based on DNA sequence data in cetaceans. Genetics 144: 1817-1833.

Mishler, B. D. 1994. Cladistics analysis of molecular and morphological data. Amer. J. Phys. Antropol. 94(1): 143-156.

Miyamoto, M.M., Kraus, F., and Ryder, O. A. 1990. Phylogeny and evolution of antlered deer determined from mitochondrial DNA sequence Proc. Natl. Acad. Sci. U.S.A. 87: 6127-6131.

Montgelard, C., Catzeflis, F. M., and Douzery, E. 1997. Phylogenetic relationships of Artiodactyls and Cetaceans as deduced from the comparison of cytochrome b and 12S rRNA mitochondrial sequences. Mol. Biol. Evol. 14(5): 550-559.

Moritz, C., and Hillis, D. M. 1996. Molecular systematics: context and controversies. In: Hillis, D. M., Moritz, C., and Marble, B. K. (eds.), Molecular systematics, 2nd. edition. Sinauer, Sunderland, MA. pp. 1-13.

Mullis, K. B. 1990. The unusual origin of the polymerase chain reaction. Scientific American, April 1990: 56-65.

Mullis, K. B., and Faloona, F. A. 1987. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. Meth. Enzymol. 155: 335-350.

Natanson, L. J., and Cailliet, G. M. 1986. Reproduction and development of the Pacific angel shark, *Squatina californica*, off Santa Barbara, California. Copeia 1986(4): 987-994.

Naylor, G. J. P., and Brown, W. M. 1997. Structural biology and phylogenetic estimation. Nature 388: 527-528.

Naylor, G. J. P., Martin, A. P., Mattison, E. G., and Brown, W. M. 1997. Interrelationships of Lamniform sharks: testing phylogenetic hypothesis with sequence data. In: Kocher, T. D., and Stepien, C. A. (eds.). Molecular systematics of fishes. Academic Press, San Diego, CA. pp. 199-218.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, NY.

Nei, M., and Tajima, F. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics 97: 145-163.

Neiva, M. A. 1992. Morfometria da albacora lage, *Thunnus albacares*, vindos do Sudoeste Equatorial no Atlântico. B.Sc. (Honours) Thesis, Universidade Federal Rural de Pernambuco, Recife, Brazil.

Nishikawa, Y., Homna, M., Ueyanagi, S., and Kikawa, S. 1985. Average distribution of larvae of oceanic species of scombroid fishes, 1956-1981. Far Seas Fish. Res. Lab. S. Ser. 12: 1-99.

Novacek, M. J. 1992. Mammalian phylogeny: shaking the tree. Nature 356: 121-125.

O'Corry-Crowe, G. M., Suydam, R. S., Rosenberg, A., Frost, K. J., and Dizon, A. E. 1997. Phylogeography, population structure and dispersal patterns of the beluga

whale *Delphinapterus leucas* in the western Nearctic revealed by mitochondrial DNA. Molecular Ecology 6: 955-970.

Ohland, D. P., Harley, E. H., and Best, P. B. 1995. Systematics of cetaceans using restriction site mapping of mitochondrial DNA. Mol. Phylog. Evol. 4(1): 10-19.

Ovenden, J. R. 1990. Mitochondrial DNA and marine stock assessment: a review. Aust. J. Mar. Freshwater Res. 41: 835-853.

Pääbo, S., Gifford, J. A., and Wilson, A. C. 1988. Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Res. 16: 9775-9787.

Palsbøll, P., Bérubé, M., Larsen, A. H., and Jørgensen, H. 1997a. Primers for the amplification of tri- and tetramer microsatellite loci in baleen whales. Mol. Ecol. 6: 893-895.

Palsbøll, P., Allen, J., Bérubé, M., Clapham, P. J., Feddersen, T. P., Hammond, P. S., Hudson, R. R., Jørgensen, H., Katona, S., Larsen, A. H., Larsen, F., Lien, J., Matilla, D. K., Sigurjónsson, J., Sears, R., Smith, T., Sponer, R., Stevick, P., and Oien, N. 1997b. Genetic tagging of humpback whales. Nature 388: 767-769.

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction. In: Hillis, D. M.; Moritz, C., and Marble, B. K. (eds.). Molecular systematics, 2nd edition. Sinauer, Sunderland, MA. pp. 205-247.

Palumbi, S. R., and Baker, C. S. 1994. Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. Mol. Biol. Evol. 11(3): 426-435.

Patterson. C. 1987. Molecules and morphology in evolution: conflict or compromise? Cambridge University Press. Cambridge. U.K.

Pepin. P.. and Carr. S. M. 1993. Morphological. meristic. and genetic analysis of stock structure in juvenile Atlantic cod (*Gadus morhua*) from the Newfoundland shelf. Can. J. Fish. Aquat. Sci. 50: 1924-1933.

Perry. E. A.. Carr. S. M.. Bartlett. S. E.. and Davidson. W. S. 1995. A phylogenetics perspective on the evolution of reproductive behavior in pagophilic seals of the northwest Atlantic as indicated by mitochondrial DNA sequences. J. Mammal. 76: 22-31.

Poey. F. 1867. Revista de los peces descritos por Poey. Rep. Fisico-Nat. Isla de Cuba 2: 153-174.

Rahn. E.. and Yesaki. M. 1976. Sumário das observações biológicas sobre o cação-anjo (*Squatina argentina*) na costa sudeste e sul do Brasil. FAO-Sudepe Documentos técnicos 18: 1-16.

Richard. K. R.. Whitehead. H.. and Wright. J. M. 1996. Polymorphic microsatellites from sperm whales and their use in the genetic identification of individuals from naturally sloughed pieces of skin. Mol. Ecol. 5: 313-315.

Rivas. L. R. 1966. Review of the *Lutjanus campechanus* complex of red snappers. Q. J. Flor. Acad. Sci. 29: 117-136.

Roff. D. A.. and Bentzen. P. 1989. The statistical analysis of mitochondrial DNA polimorphisms: χ^2 and the problem of small samples. Mol. Biol. Evol. 6: 539-545.

Russo, C. A. M. 1997. Efficiencies of different statistical tests in supporting a known vertebrate phylogeny. Mol. Biol. Evol. 14 (10): 1078-1080.

Russo, C. A. M., Takezaki, N., and Nei, M. 1996. Efficiencies of different genes and different tree-building methods in recovering a known vertebrate phylogeny. Mol. Biol. Evol. 13(3): 525-536.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

Saitou, N., and Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4): 406-425.

Salles, R. 1997. Identificação dos estoques do pargo, *Lutjanus purpureus* Poey, nas regiões Nordeste e Norte do Brasil, entre 43°W e 49°W. M.Sc. Thesis. Universidade Federal do Ceará, Fortaleza, Brazil.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74: 5463-5467.

Sarver, S. K., Freshwater, D., and Walsh, P. J. 1996. Phylogenetic relationships of western Atlantic snappers (Family Lutjanidae) based on mitochondrial DNA sequences. Copeia 1996(3): 715-721.

Schaefer, K. M. 1991. Geographic variation in morphometric characters and gill-raker counts of yellowfin tuna *Thunnus albacares* from the Pacific Ocean. Fish. Bull. 83: 289-297.

Schaefer, K. M. 1992. An evaluation of geographic and annual variation in morphometric characters and gill-raker counts of yellowfin tuna *Thunnus albacares* from the Pacific Ocean. Inter-Am. Trop. Tuna Comm. Bull. 20: 133-163.

Schaefer, M. B., and Walford, L. A. 1950. Biometric comparations between yellowfin tunas (*Neothunnus*) of Angola and the Pacific coast of Central America. U.S. Fish and Wildl. Serv. Fish. Bull. 51: 425-443.

Scoles, D. R., and Graves, J. E. 1993. Genetic analysis of the population structure of yellowfin tuna, *Thunnus albacares*, from the Pacific Ocean. Fish. Bull. 91: 690-698.

Secchi, E. R., Wang, J. Y., Murray, B., Rocha-Campos, C. C., and White, B. N. 1998. Population differences between franciscana, *Pontoporia blainvillei*, from two geographical locations as determined by sequences of mtDNA control region. Abstracts of the World Marine Mammal Science Conference. Monaco, Society of Marine Mammalogy and the European Cetacean Society. p.122.

Shinohara, M., Domingo-Roura, X., and Takenaka, O. 1997. Microsatellites in the bottlenose dolphin *Tursiops truncatus*. Mol. Ecol. 6: 695-696.

Silva, K. G. 1996. Estudo comparativo dos parâmetros populacionais da reprodução dos cações-anjo *Squatina argentina*, *S. guggenheim*, e *S. occulta*, no sul do Brasil. Master thesis. Fundação Universidade de Rio Grande, Rio Grande do Sul, Brazil.

Siciliano, S. 1994. Review of small cetaceans and fishery interactions in coastal waters of Brazil. Rep. Int. Whal. Commn (Special Issue 15): 241-250.

Simões-Lopes, P.C. 1988. Ocorrência de uma população de *Sotalia fluviatilis* Gervais, 1953 (Cetacea, Delphinidae) no limite sul de sua distribuição, Santa Catarina, Brasil. Biotemas 1: 57-62.

Simon, C., Frati, F., Beckenback, A., Crespi, B., Liu, H., and Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651-701.

Simpson, G. G. 1961. Principles of animal taxonomy. Columbia University Press, New York, NY.

Slijper, E. 1962. Whales. Hutchinson, London, UK.

Slatkin, M. 1985. Rare alleles as indicators of gene flow. Evolution 39: 53-65.

Smith, J. F., and Systma, K. L. 1994. Molecules and morphology congruence of data in Columnea (Gesneriaceae). Plant Syst. Evol. 193: 37-52.

Solé-Cava, A. M., Vooren, C. M., and Levy, J. A. 1983. Isozymic differentiation of two sibling species of *Squatina* (Chondrichthyes) in south Brazil. Comp. Biochem. Physiol., B.75b (2): 355-358.

Solé-Cava, A. M., and Levy, J. A. 1987. Biochemical evidence for a third species of angel shark off the East coast of South America. Biochem. Syst. Ecology. 15(1): 139-144.

Stern, D. L. 1994. A phylogenetic analysis of soldier evolution in the aphid family Hormaphididae. Proc. Royal Soc. Lond., B. Biol. Sci. 256: 203-209.

Swofford, D. L. 1993. PAUP: Phylogenetic Analysis Using Parsimony, version 3.1.: Manual. Illinois Natural History Survey, Chapaign, IL.

Swofford, D. L. 1997. PAUP: Phylogenetic Analysis Using Parsimony, versions 4.0d59-4.0d61. Computer program: test versions.

Swofford, D. L., Olsen, G. J., Waddell, P. J., and Hillis, D. M. 1996. Phylogenetic inference. In: Hillis, D. M., Moritz, C., and Mable, B. K. Molecular Systematics, 2nd. edition. Sinauer, Sanderland, MA.

Taylor, P. G. 1996. Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. Mol. Biol. Evol. 13(1): 283-285.

Thewissen, J. G. M., and Hussain, S. T. 1993. Origin of underwater hearing in whales. Nature 361: 444-445.

Thewissen, J. G. M., Hussain, S. T., and Arif, M. 1994. Fossil evidence for the origin of aquatic locomotion in Archaeocete whales. Science 263: 210-212.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. 1996. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. Science 272: 1136-1144.

Utter, F. 1991. Biochemical genetics and fisheries management: an historical perspective. J. Fish. Biol. 39 (Suppl. A): 1-20.

Valsecchi, E., and Amos, W. 1996. Microsatellites markers for the study of cetacean populations. Mol. Ecol. 5: 151-156.

Valsecchi, E., Palsbøll, P., Hale, P., Glockner-Ferrari, D., Ferrari, M., Clapham, P., Larsen, F., Mattila, D., Sears, R., Sigurjonsson, J., Brown, M., Corkeron, P., and Amos, B. 1997. Microsatellite genetic distances between oceanic populations of the humpback whale (*Megaptera novaeangliae*). Mol. Biol. Evol. 14(4): 355-362.

Van Bénéden, E. 1875. Memoire sur un dolphin nouveau de la Baie de Rio de Janeiro. Memoires de l'Academie Royale de Sciences Belgique 41: 2-44.

Van Valen, L. 1966. Deltatheridia, a new order of mammals. Bull. Amer. Mus. Nat. Hist. 132: 1-126.

Van Valen, L. 1968. Monophyly or diphyly in the origin of whales. Evolution 22: 37-41.

Vergara, R. R. 1980. Consideraciones filogeneticas sobre las especies Cubanas genero *Lutjanus* (Lutjanidae, Perciformes, Teleostei). Informe Cientifico-Técnico 113: 1-39.

Vidal, O., Barlow, J., Hurtado, L.A., Torre, J., Cendón, P., and Ojeda, Z. 1997. Distribution and abundance of the Amazon river dolphin (*Inia geoffrensis*) and the tucuxi (*Sotalia fluviatilis*) in the upper Amazon River. Mar. Mammal Sci. 13(3): 427-445.

Vis, M. L., Carr, S. M., Bowering, W. R., and Davidson, W. S. 1997. Greenland halibut (*Reinhardtius hippoglossoides*) in the North Atlantic are genetically homogeneous. Can. J. Fish. Aquat. Sci. 54: 1813-1821.

Vogler, A. P., and Welsh, A. 1997. Phylogeny of North American *Cicindela* tiger beetles inferred from multiple mitochondrial DNA sequences. Mol. Phylogen. Evol. 8(2): 225-235.

Vooren, C. M., and Silva, K. G. 1991. On the taxonomy of the angel sharks from southern Brazil, with the description of *Squatina occulta* sp.n. Rev. Brasil. Biol. 51(3): 589-602.

Ward, R. D., Elliott, N. G., Innes, B. H., Smolenski, A. J., and Grewe, P. M. 1997. Global population structure of yellowfin tuna, *Thunnus albacares*, inferred from allozyme and mitochondrial DNA variation. Fish. Bull. 95(3): 566-575.

Webb, S. D., and Taylor, B. E. 1980. The phylogeny of hornless ruminants and a description of the cranium of *Archaeomeryx*. Amer. Museum Nat. Hist. Bull. 167: 117-158.

Wiley, E. O. 1981. Phylogenetics: the theory and pratice of phylogenetics systematics. John Wiley & Sons, New York, NY.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. S. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-813.

Wilson, A. C., Cann, R. L., Carr, S. M., George Jr., M., Gyllensten, U. B., Helm-Bychowski, K., Higuchi, R. G., Palumbi, S. R., Prager, E. M., Sage, R. D., and

Stoneking, M. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Soc. 26: 375-400.

World Scientific Report. 1993. Unesco. Paris.

Wourms, J. P. 1977. Reproduction and development in chondrichthyan fishes. Amer. Zool. 17: 379-410.

Wourms, J. P., and Demski, L. S. The reproduction and development of sharks, skates, rays and ratfishes: introduction, history, overview and future prospects. Environm. Biol. Fish. 38(1-3): 7-21.

Yanez, E., and Barbieri, M. A. 1980. Analyse de la prise par unité d'effort saisonnière et de l'evolution de l'indice gonado somatique de la peche palangriere (1956-1977) et de surface (1969-1978) du yellowfin (*Thunnus albacares*) de l'Atlantique. Rec. Doc. Scient. ICCAT 9(1): 76-91.

Xu, X., and Arnason, U. 1994. The complete mitochondrial DNA sequence of the horse, *Equus caballus*: extensive heteroplasmy of the control region. Gene 148: 357-362.

Xu, X., Janke, A., and Arnason, U. 1996. The complete mitochondrial DNA sequence of the greater Indian rhinoceros, *Rhinoceros unicornis*, and the phylogenetics relationship among Carnivora, Perissodactyla, and Artiodactyla (+ Cetacea). Mol. Biol. Evol. 13(9): 1167-1173.