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Population genetics of selected species of sharks

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POPULATION GENETICS OF SELECTED SPECIES OF SHARKS

A Dissertation
Presented to
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In Partial Fulfillment
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by

Edward Jay Heist

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

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ABSTRACT

Molecular genetic techniques were used to elucidate genetic population structure in three species of sharks, the coastal sandbar shark (*Carcharhinus plumbeus*) and Atlantic sharpnose shark (*Rhizoprionodon terraenovae*), and the pelagic shortfin mako (*Isurus oxyrinchus*). Allozyme analysis and analysis of restriction fragment length polymorphisms (RFLP) of mitochondrial DNA (mtDNA) were used to test the null hypothesis that the mid-Atlantic Bight and the Gulf of Mexico sandbar sharks consist of a single gene pool. RFLP analysis of mtDNA was used to determine the pattern and level of genetic divergence in the sandbar shark between the western North Atlantic and the Eastern Indian Ocean, and within the entire species range of the cosmopolitan shortfin mako and the Atlantic sharpnose shark.

No significant genetic divergence was detected in the sandbar shark between the mid-Atlantic Bight and Gulf of Mexico. Genetic variation was extremely low but homogeneously distributed. A significant degree of genetic divergence was detected between North Atlantic and Australian sandbar sharks. All Australian sandbar shark mtDNAs were fixed for alleles other than those detected in the North Atlantic.

The hypothesis that the shortfin mako comprises a single panmictic population was rejected. The overall probability of drawing samples with such disparate allele frequencies from a single gene pool was <0.001. The only barrier to gene flow detected appeared to be the equatorial Atlantic. Samples from Brazil, Australia, and California were not significantly different from each other, however all three were significantly different from the North Atlantic sample. The shortfin mako exhibited a considerably higher level of genetic variation than the sandbar shark.

The Atlantic sharpnose shark did not exhibit significant differences in allele frequency throughout its range. The level of genetic variation detected in mtDNA was intermediate to that in the sandbar shark and the shortfin mako.

POPULATION GENETICS OF SELECTED SPECIES OF SHARKS

CHAPTER ONE

GENERAL INTRODUCTION

A) Shark Fisheries and Conservation Issues

During the last decade a great increase in shark harvest has occurred in the territorial waters of the United States (Anon, 1993, Holts, 1988; Parrack, 1990), as well as many other parts of the world (Casey and Kohler, 1992; Stevens, 1992). A major stimulus to the expanding fishery has been the growing acceptance of shark meat in the diets of many people (Hoff and Musick, 1990), coupled with the decline of more traditional finfish fisheries. The rapid evolution of a large-scale fishery for a previously under-utilized resource creates the risk that stocks may become overfished before data can be obtained for the implementation of proper management. This danger of overfishing is especially threatening to species with low fecundities, slow growth rates, and long maturation times, such as the sharks currently involved in the U.S. and other commercial fisheries (Hoff and Musick 1990). Holden (1973, 1974), referring to these life-history characteristics, argued that long-term fisheries of sharks were possible only if care was taken to address the relevant biological

characteristics of the species involved.

In consideration of the risk of over-exploitation of sharks, the United States National Marine Fisheries Service (NMFS) implemented a fishery management plan for sharks in the Northwest Atlantic waters within the exclusive economic zones enforced by the United States (Anon, 1993). This plan suffers from a lack of basic information concerning the movements of important species within and beyond the jurisdictional area of the regulating agency. The plan divides sharks in the management area into three categories for regulation purposes: large coastal species, small coastal species, and pelagic species. Under the current plan only catches of the large coastal sharks are being regulated. Pelagic species (chiefly makos and blue shark) are not regulated because it is known that they travel great distances across international borders, and hence are sensitive to exploitation outside of the 200 mile jurisdiction established by the 1976 Magnuson Act. Small coastal species are not being managed because analysis of current data indicates that catch rates are not significantly greater than maximum sustainable yield, and that the higher fecundity of small coastal species (e.g. sharpnose and blacknose) would allow them to recover rapidly if fishing pressure were to cease (Anon, 1993).

In this dissertation the techniques of molecular population genetics are used to investigate the genetic

population structure of three important species of sharks, one large coastal, one small coastal, and one pelagic. The two species chosen for this study are the sandbar shark (*Carcharhinus plumbeus*) and the shortfin mako (*Isurus oxyrinchus*). The sandbar shark is the most important species in the US coastal longline shark fishery, comprising approximately 80% of all landings by weight (Anon, 1993).

Although less common than the blue shark (*Prionace glauca*), the shortfin mako has a higher market value and is the most economically important pelagic shark worldwide, supporting commercial and recreational fisheries throughout the temperate to tropical oceans (Casey and Kohler, 1992; Holts, 1988; Stevens, 1992). Over the last few decades there has been a great increase of commercial and recreational harvest for this species in the North Atlantic (Casey and Kohler, 1992) and in the North Pacific (Holts, 1988).

B) Molecular Population Genetics

When a population becomes subdivided into two or more subpopulations (or stocks), neutral allele frequencies at variable loci drift at a rate that is inversely proportional to the effective size of the subpopulation. If isolation is complete, genetic characters continue to diverge until the subpopulations have fixed allelic differences at multiple

loci. Migration between populations (defined here as the mating of an individual from one subpopulation within another subpopulation) tends to reduce the effect of drift by homogenizing allele frequencies between subpopulations. Eventually an equilibrium is attained between migration and drift whereby an equilibrium level of genetic divergence will be maintained between regions. The magnitude of genetic divergence will be determined by the absolute number of migrants between populations (Allendorf and Phelps, 1981).

Historically the most important technique developed to examine genetic variability within and among populations has been allozyme electrophoresis (Ryman and Utter, 1987). This procedure uses starch gels to separate intact enzymes in an electric field based on the ionic charge and shape resulting from the amino acid sequence of the protein, then uses histochemical stains to visualize the migration of the enzyme. Substitutions in the amino acid sequence of enzymes, which are the direct result of changes in the DNA sequence within the gene for the enzyme, often result in different migration rates (or directions) for the active molecule. Individuals are scored as either homozygotes or heterozygotes at variable loci, and allele frequencies are determined for each locus. There are histochemical stain recipes published for over one hundred allozyme loci, and many recent studies utilize thirty or more different loci to

examine population structure. Allozyme electrophoresis was the first technique that allowed for direct scoring of many loci in natural populations, and since its inception in the 1960s more has been learned about the genetic structure of populations using allozymes than with any other procedure.

Although allozyme electrophoresis is a very powerful tool, the procedure has several drawbacks. Because only active enzyme molecules react to the histochemical stains, very high tissue quality is required. Degraded tissue shows no activity, or produces artifactitious "sub-bands". Different tissues exhibit different enzymatic activities, so usually several tissue types are required in order to score a large suite of loci. Finally, a majority of the genetic variability that exists at the DNA level is undetected by the allozyme technique because the redundancy of the DNA code prevents many mutations from changing the amino acid sequence of a protein, and many of the amino acid substitutions that do occur have no detectable effect on the migration of the enzyme (Lewontin and Hubby, 1966). Previous studies of allozymes in sharks have detected low degrees of intraspecific variation (Lavery and Shaklee, 1989; Smith, 1986), thereby limiting the resolving power of interpopulation divergence.

The useful data generated by allozyme analysis are the frequencies of alleles at polymorphic loci between locations. When two or more populations with different

allele frequencies are combined into a single sample, the result is an excess of homozygotes relative to Hardy-Weinberg expectations. Simple chi-square analysis can be performed to test whether a sample conforms to Hardy-Weinberg expectations. The excess of homozygotes in the presence of genetic population structure is called a "Wahlund effect" after Wahlund (1928). The statistic used to evaluate this excess is Wright's F_{st} statistic (Wright, 1978), which is calculated over all polymorphic loci.

A more recent technique for the investigation of population structure is the analysis of mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs). This method utilizes bacterial restriction enzymes that recognize specific palindromic DNA sequences and cleave double stranded DNA. When a restriction enzyme is used to cleave purified mtDNA, a population of fragments is produced such that the number of fragments is equal to the number of specific palindromic sequences in the mtDNA molecule. The fragments are separated electrophoretically by size on an agarose gel and then scored either by direct visualization of ethidium bromide incorporated DNA, by autoradiography of radio-labeled DNA, or by transferring to a filter and hybridizing with labeled DNA.

The major advantage that RFLP analysis of mtDNA has over allozyme analysis is that every mutation that occurs in specific restriction sites is detectable. Through the

technique of Southern blotting using labelled purified mtDNA from a high quality tissue source it is possible to obtain mtDNA RFLP data from partially degraded samples, making it feasible to obtain tissue from geographic locations where the collection of samples for allozyme analysis would be impractical.

RFLP data can be treated in several ways. Composite haplotypes can be considered as alternate alleles at a single mtDNA locus, and the hypothesis that samples could have been drawn from a single gene pool can be tested. Based on the number of nucleotides sampled and the diversity detected the percentage of nucleotide sites that differ between individuals can be estimated. The amount of nucleotide diversity detected in a study can be partitioned into within sample and between sample portions so that a qualitative determination of the degree of isolation between locations can be assessed.

Many researchers are currently using RFLP analysis of mtDNA to address problems where allozymes would previously have been used, and there are reasons to expect that the different methods may not suggest the same conclusions. Because animal mtDNA is predominately clonally inherited from the maternal parent (Wilson et al., 1985), individuals possess a haploid genotype, and the genotype of the paternal parent has no contribution. This results in at least a four-fold decrease in effective population size for

mitochondrial as opposed to nuclear genes (Birkey et al., 1983). Therefore genetic differentiation should occur more rapidly in mitochondrial DNA. Species whose sexes exhibit different migrational tendencies (such as many species of sharks) may have different forces acting on diploid nuclear genes that undergo recombination than on haploid clonal genes.

In this dissertation allozyme electrophoresis and analysis of RFLPs from mtDNA are used to investigate the genetic population structure of three important species, one large coastal, one pelagic, and one small coastal. The species chosen for this study are the sandbar shark (*Carcharhinus plumbeus*), the shortfin mako (*Isurus oxyrinchus*), and the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*). The sandbar has numerous allopatric populations throughout the world's warm-temperate and subtropical oceans (Compagno, 1984). The shortfin mako has an apparently continuous tropical and subtropical distribution. The Atlantic sharpnose shark is found only within the western North Atlantic, but occupies a considerable latitudinal range.

CHAPTER TWO

Population Genetics of the Sandbar Shark (*Carcharhinus plumbeus*) in the Gulf of Mexico and Mid-Atlantic Bight.

The sandbar shark (*Carcharhinus plumbeus*) is a large (maximum adult length > 2 m), coastal species that has numerous allopatric populations throughout the warm temperate and sub-tropical oceans (Compagno, 1984). The species ranges from Massachusetts to Brazil along the western North Atlantic coast, including the Caribbean Sea (Compagno, 1984). The sandbar shark is the most common species of large shark in the United States' coastal Atlantic waters as well as the Gulf of Mexico (Springer, 1960), and is one of the most important sharks in the United States' shark longline fishery (Musick et al., 1993).

In the western North Atlantic, the sandbar shark undergoes seasonal migrations, however the two sexes become segregated as adults. Male/female ratios are nearly equal at birth (Springer, 1960; Clark and von Schmidt, 1965), but larger individuals captured in the mid-Atlantic Bight are almost exclusively female with larger males remaining to the south and farther offshore. The largest pupping grounds for the sandbar shark occur in the mid-Atlantic Bight from New York to Cape Hatteras and especially in Chesapeake Bay

(Musick and Colvocoresses, 1988). Young sandbar sharks of both sexes are common in inshore regions of these latitudes during the summer but move offshore and southward during the winter, presumably to the edge of the Gulf Stream off North Carolina (Compagno, 1984). Larger individuals (age five years and older) migrate south to the Gulf of Mexico, as revealed by the dozens of individuals tagged in the mid-Atlantic Bight that were later recaptured in the Gulf of Mexico (Casey and Kohler, 1990).

Movement of individuals throughout a population's range does not preclude genetic divergence within the putative population. Springer (1960) suggested that the sandbar sharks of the western North Atlantic may contain two separate breeding populations, a major one off the mid-Atlantic coast of North America and a minor one in the western Gulf of Mexico. If Springer's suggestions are correct, then the sandbar sharks that occupy the Gulf of Mexico may include a mixture of stocks from both pupping grounds.

Differences in the migrational tendencies of males and females may have a differential effect on mitochondrial genes versus nuclear genes. If female sandbar sharks have separate natal pupping zones in the western North Atlantic, it is possible that mtDNA haplotype frequencies have diverged between pupping locations regardless of the movements of the males. Meylan et al. (1990) found that

although green sea turtles from various natal beaches have overlapping feeding grounds, frequencies of mtDNA genotypes differed greatly between females from different natal islands. Karl et al. (1992) showed that Mendelian characteristics in green turtles showed less divergence, indicating male-mediated gene flow. The Gulf of Mexico may contain mixtures of sandbar sharks from various pupping areas and therefore haplotypes common in other regions (e.g. the Caribbean Sea) may be present at low frequencies within the Gulf of Mexico but completely absent in the Chesapeake Bay. Bi-parental inheritance of allozyme characters will prevent divergence if even a small number of males (i.e. > one per generation) mate with females from other pupping grounds (Wright, 1978).

Several studies have used molecular techniques to analyze population genetics of temperate and subtropical marine fishes between the Gulf of Mexico and the Atlantic coast of North America (reviewed by Avise, 1992). The subtropical southern point of the Florida peninsula today serves as a barrier to gene flow between populations of many temperate species of marine fishes and invertebrates. Some species with limited migrational tendencies, (e.g. horseshoe crab, black sea bass) exhibit genetic divergence between the Gulf of Mexico and southeastern U.S. coast (Saunders et al., 1986; Bowen and Avise, 1990), whereas other highly migratory species of fishes (e.g. American eel, bluefish) display no

significant genetic divergence (Avise et al., 1986; Graves et al., 1992). During the heights of the Wisconsin glaciation, ending 10,000 to 15,000 years before present, temperate populations that are now subdivided may have mixed in a refugia in the Caribbean Sea or southern Gulf of Mexico. Current levels of genetic divergence may have been influenced by both current patterns of gene flow and historical differences in species distribution. Our study uses both allozyme electrophoresis and RFLP analysis of mtDNA to test the null hypothesis that sandbar sharks from the Gulf of Mexico and the Chesapeake Bay and adjacent coastal waters represent a single gene pool.

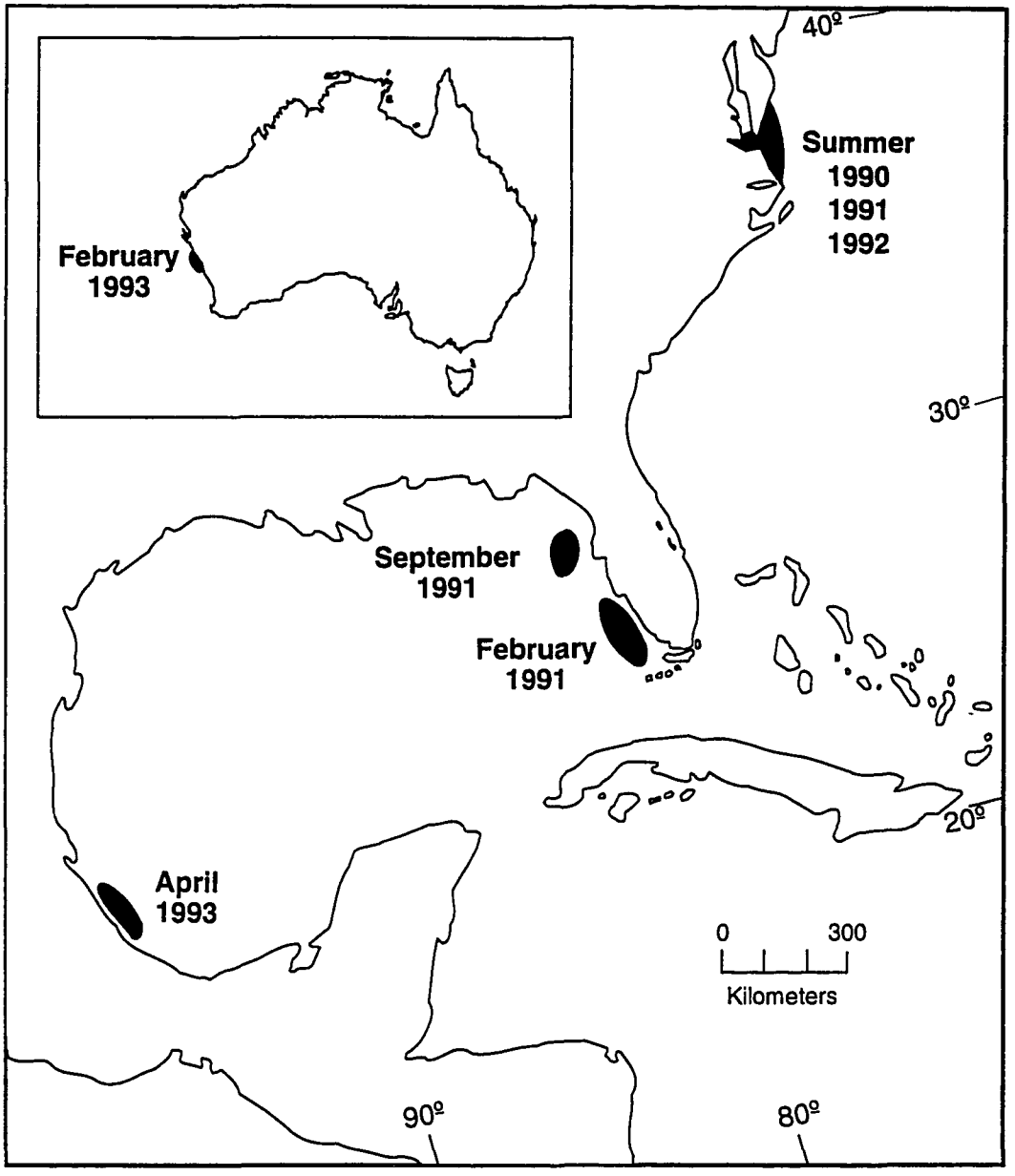
METHODS

Sample collection.-Sandbar sharks were captured with research longlines from the Chesapeake Bay and adjacent coastal waters of Virginia ('Bay'), n=173, as part of the ongoing shark research program of the Virginia Institute of Marine Science (VIMS). Sharks were also collected from the Gulf of Mexico along the southwestern coast of Florida ('Gulf'), n=222, aboard a commercial shark fishing vessel, and from artisanal longline vessels from Veracruz, Mexico, n=5, (Figure 1). The temporal sampling strategy involved the collection of Bay specimens separately during three consecutive summers, 1990-1992, Florida 'Gulf' specimens in February and September, 1991, and Mexican 'Gulf' samples in

April, 1993. The majority of the Bay specimens were juveniles less than 1 m total length and were presumably less than five years of age. These sharks were apparently too young to have made a migration to the Gulf of Mexico (Casey et al., 1985). The Gulf samples were from adult and sub-adult sharks greater than 1.5 m total length. One individual captured in the Gulf of Mexico in 1991 had been tagged in the Atlantic off Montauk Pt., USA (J. Casey, cooperative shark tagging program, pers. comm.). Heart, kidney, and white muscle tissue from each of the specimens were removed and frozen immediately in liquid nitrogen, except for the Mexican samples that were collected as whole hearts, chilled on wet ice, and temporarily stored in a household freezer (-20°C) for five to ten days. After transport to the lab, tissues were stored at -70 °C until analyzed.

Allozyme electrophoresis.-Homogenates were separately prepared with heart, kidney, and white muscle tissues by grinding with a mechanical tissue homogenizer in approximately two volumes of ice-cold grinding buffer (100 mM tris, 1 mM EDTA, pH 7.0). The resultant preparation was centrifuged at 1200 g for 10 min, and the supernatant decanted and stored at -70 °C prior to electrophoresis. The homogenates were subjected to electrophoresis on 12% horizontal starch gels (Starch Art Inc.) using the apparatus

Figure 1. Locations and dates of collection of the sandbar shark (*Carcharhinus plumbeus*).


40º
Summer
1990
1991
1992
February
1993
30º
September
1991
February
1991
20º
April
1993
0
300
Kilometers
90º
80º

of Murphy et al. (1990) for 16-20 hr at 2.5 V/cm. Extracts of each tissue type were separated electrophoretically on eight different buffer systems and stained with approximately 40 histochemical stains to determine a suite of tissue/buffer/stain combinations that adequately resolved products of a set of presumptive allozyme loci (Table 1). All buffers and histochemical stains followed Murphy et al. (1990) except for the triethanolamine-citrate (TRIC) buffer of Clayton and Tretiak (1972). Wright's F statistics (Wright, 1978) and chi-square analysis of Hardy-Weinberg expectations were calculated using the BIOSYS-1 program of Swofford and Selander (1981).

Mitochondrial DNA RFLP Analysis.-Mitochondrial DNA was isolated from heart tissue using cesium chloride density-gradient ultracentrifugation following Lansman et al. (1981). Aliquots of mtDNA were digested using twelve restriction enzymes following the manufacturers' instructions. Fragments were separated on 1.0% horizontal agarose gels run at 2V/cm overnight and visualized following one of two techniques. When tissue was abundant and freshly frozen, mtDNA fragments were radiolabelled with ^{35}S-labelled nucleotides prior to electrophoresis using the large (Klenow) fragment of DNA polymerase I (Sambrook et al., 1989). After electrophoresis the gels were treated with a scintillation enhancer, dried, exposed to x-ray film for 3

to 6 days, and visualized autoradiographically. For those specimens with small tissue samples or with partially degraded tissue, fragments were transferred after electrophoresis to a nylon membrane via Southern transfer following the protocols of Sambrook et al. (1989). The fragments were probed with highly purified shortfin mako (*Isurus oxyrinchus*) mtDNA nick-translated with biotin-7 dATP and visualized using the BRL BlueGene Nonradioactive Nucleic Acid Detection System.

Fragment patterns were scored for each restriction enzyme and each individual was assigned a composite genotype based on the fragment patterns for all enzymes. Each polymorphism (RFLP) could be explained by the gain or loss of one (or in one case two) restriction sites relative to the common pattern. The nucleon (genotypic) diversity was calculated for each sample and for the composite of both samples following Nei (1987). Nucleotide sequence diversity was calculated following the site approach of Nei and Li (1979). Chi-square significance of the difference in genotypic frequencies between samples was computed using the randomization protocol of Roff and Bentzen (1989).

RESULTS

Allozyme electrophoresis.-Products of 27 presumptive loci were scored for a minimum of one hundred individuals (Table 1). Products of approximately 20 other presumptive loci

were observed in a smaller number of individuals, but these loci were not resolved clearly. Twenty-five of 27 loci were monoallelic in all samples. Overall 3.58% of the loci assayed were variable with an average heterozygosity among loci and samples equal to 0.005.

Only one locus, Ldh-B, was sufficiently polyallelic to be used to test population structure hypotheses. A second locus, mMdh-A, produced a single heterozygote in 116 individuals. Aspartate transaminase (sAta-A) appeared to have a high degree of heterozygosity, but was one the loci excluded from the analysis because of poor resolution.

The overall frequency of the most common Ldh-B allele was 0.928, and frequencies were very similar for the Bay (0.922) and Gulf (0.932) samples. Observed Ldh-B genotypic frequencies conformed to expected Hardy-Weinberg proportions in both the Bay ($\chi^2<0.001$, $p>0.98$) and Gulf ($\chi^2=1.125$, $p>0.25$) populations. A contingency chi-square analysis indicated that the observed genotypic distributions did not differ significantly from the sampling of a single population with an allele frequency of 0.928 ($\chi^2=1.344$, $p>0.5$).

Analysis of mitochondrial DNA.-Mitochondrial DNA restriction patterns were determined for 95 sandbar sharks using twelve restriction enzymes (Appendix 1, Table 2). Ten of twelve restriction enzymes revealed invariant fragment patterns in

all individuals. The other two restriction enzymes yielded a total of five haplotypes (Appendix 1), four of which occurred at very low frequency (<0.04) and differed from the common pattern by one or two restriction sites. Not all individuals were scored for all enzymes; 3.7% of the cells in the restriction fragment pattern matrix were unscored due to the exhaustion of tissue samples. However, individuals with missing restriction pattern data were assumed to possess the common pattern only for those enzymes in which no variants were detected after scoring a minimum of 85 individuals. The approximate size of the sandbar shark mitochondrial molecule was estimated at 16.7 kilobase pairs (Table 2). Twelve restriction enzymes with four, five, and six-base recognition sites produced an average total of 58 restriction fragments per individual. The mean number of bases surveyed was 301, or 1.79% of the mitochondrial DNA molecule.

The composite nucleon diversity was very low (0.161) with 87 of 95 individuals sharing the common genotype (Table 2). The Gulf sample had a slightly higher nucleon diversity (n.d.) than the Bay sample (n.d. = 0.22 and 0.11, respectively).

The corrected mean nucleotide sequence divergence between the Bay and Gulf samples was less than 0.001%, compared to the within sample nucleotide sequence diversities of 0.026% for the Bay sample and 0.046% for the

Gulf sample. The overall mean nucleotide sequence diversity was 0.036%. Of the five mitochondrial genotypes detected, four were encountered in both samples, and the remaining genotype was present in only a single individual in the Gulf sample (Table 2). All five individuals from Veracruz possessed the common genotype, and were thus grouped with the Florida samples into a common 'Gulf' sample. The Roff and Bentzen (1989) randomization analysis produced a chi-square value higher than the observed value in 812 of 1000 randomizations of the genotype frequency data, suggesting that the null hypothesis of a single gene pool could not be rejected ($p=0.81$).

Table 1. Conditions and results of allozyme analysis of the sandbar shark (*Carcharhinus plumbeus*).

Enzyme (code number)[a]	Locus	Tissue[b]	Buffer[c]	N Bay	N Gulf
Aconitate hydratase (4.2.1.3)	Acon-A	H	TCIII	36	66
Adenylate kinase (2.7.4.3)	Ak-2	M	TCII	36	70
α-Manosidase (3.2.1.24)	αMan-A	K	TE	65	57
α-Glucosidase (3.2.1.20)	αGlus-A	K	TCIII	36	68
Creatine kinase (2.7.3.2)	Ck-C	H	TE	42	72
Dihydrolipoamide dehydrogenase (1.8.1.4)	Ddh-A	K	TCIII	39	64
Esterase (3.1.1.-)	Est-1	K	Borate	54	74
Esterase (3.1.1.-)	Est-2	K	Borate	57	74
Esterase (3.1.1.-)	Est-3	K	Borate	57	74
Formaldehyde dehydrogenase (1.2.1.1)	Fdh-A	K	TRIC	36	74
Glycerol-3-phosphate dehydrogenase (1.2.1.8)	G3pdh-A	M	TCIII	40	66
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6pdh-A	K	TCIII	36	70
L-Iditol Dehydrogenase (1.1.1.14)	Iddh-A	K	TCIII	31	73
L-Lactate dehydrogenase (1.1.1.27)	Ldh-A	H or K	TRIC	173	222
L-Lactate dehydrogenase (1.1.1.27)	Ldh-B	H or K	TRIC	173	222
Malate dehydrogenase (1.1.1.37)	mMdh-A	H	CAPM	42	70
Malate dehydrogenase (1.1.1.37)	sMdh-A	H	CAPM	42	70
Malic enzyme (1.1.1.40)	mMdhp-A	H or M	CAPM	42	74
Malic enzyme (1.1.1.40)	sMdhp-A	H or M	CAPM	42	74
Octanol dehydrogenase (1.1.1.73)	Odh-A	K	TE	54	67
Peptidase (3.4.-.-)	Pep-1[d]	K	LiOH	54	64
Peptidase (3.4.-.-)	Pep-2[d]	K	LiOH	54	52
Peptidase (3.4.-.-)	Pep-3[d]	K	LiOH	54	52
Peptidase (3.4.-.-)	Pep-4[d]	K	LiOH	54	59
Phosphogluconate dehydrogenase (1.1.1.44)	Pgdh-2	K	TCIII	54	49
Superoxide dismutase-1 (1.15.1.1)	Sod-1	H	CAPM	36	64
Superoxide dismutase-2 (1.15.1.1)	Sod-2	H	CAPM	36	64

Table 1. (Continued)

a) IUBMBNC (1992)

b) H=Heart, K=Kidney, M=White Muscle.

c) a) Borate (continuous) = pH 8.6,
b) CAPM = Citric acid Aminopropyl morpholine, pH 6.0,
c) LiOH = Lithium-borate/tris citrate, pH 8.3,
d) Tris-citrate II, pH 8.0,
e) Tris-citrate III, pH 7.2
f) Tris-EDTA, pH 9.6.

d) Four zones of peptidase activity were observed using three peptide substrates. Presumptive loci are listed in order of increasing anodal mobility (Pep-1 = Leu-Pro, Pep-2 = Leu-Gly-Gly, Pep-3 = Leu-Gly-Gly, Pep-4 = Ala-Met).

Table 2. Mitochondrial restriction patterns observed, genotypes designated, and number of genotypes observed in the sandbar shark (*Carcharhinus plumbeus*) from the Chesapeake Bay region and the Gulf of Mexico. Enzymes scored (left to right) are *Ava* I, *Ava* II, *Ban* I, *Bcl* I, *Bgl* I, *Dra* I, *Hae* II, *Hinc* II, *Hind* III, *Nru* I, *Sca* I, *Xho* I.

Genotype	Pattern	N
1	AAAAAAAAAAAA	87
2	ABAAAAAAAAAA	3
3	ACAAAAAAAAAA	2
4	ADAAAAAAAAAA	2
5	AAAAAAAABAAA	1
Total		95

CHESAPEAKE BAY:

	GENOTYPE					
Time of Collection	1	2	3	4	5	TOTAL
Summer 1990	14	0	1	0	0	15
Summer 1991	21	1	0	0	0	22
Summer 1992	14	0	0	1	0	15
Total:	49	1	1	1	0	52

GULF OF MEXICO:

	GENOTYPE					
Location/Time of Collection	1	2	3	4	5	TOTAL
Florida, Feb. 1991	13	2	1	0	0	16
Florida, Sep. 1991	20	0	0	1	1	22
Veracruz, April 1993	5	0	0	0	0	5
Total:	38	2	1	1	1	43

Table 2. continued

	HAPLOTYPE DIVERSITY	MEAN NUCLEOTIDE SEQUENCE DIVERSITY
CHESAPEAKE BAY	0.11	0.026%
GULF OF MEXICO	0.22	0.046%

COMBINED HAPLOTYPE DIVERSITY = 0.161

CORRECTED MEAN NUCLEOTIDE SEQUENCE DIVERGENCE = <0.001%

DISCUSSION

Sandbar sharks are characterized by relatively low levels of genetic variation. Smith (1986) reported a range of allozyme heterozygosities of 0.011 to 0.037 for seven species of sharks, with the highest heterozygosity belonging to blue shark, *(Prionace glauca)*, the only carcharhinid in that study. Lavery and Shaklee (1989) reported allozyme heterozygosities of 0.035 and 0.037 for two species of *Carcharhinus* in Australia. The heterozygosity of 0.005 determined for *C. plumbeus* in this study is nearly an order of magnitude less than that of the above published accounts of heterozygosity in carcharhinids, and is low compared to typical values for other fishes (see summary in Smith and Fujio, 1982). The nucleon diversity of 0.16 and the nucleotide sequence diversity of 0.036% for the sandbar shark are very low compared to the numerous values published for teleost taxa (Avise et al., 1989; Ovenden, 1990).

Neither allozyme nor mtDNA analysis provide evidence to reject the null hypothesis that the samples share a single gene pool. Although genetic variability was extremely low for both allozymes and mtDNA, the similar frequencies of Ldh-B alleles and the occurrence of the same rare mtDNA alleles in both locations support the hypothesis that

samples were collected from a single population. No evidence of the contribution to the stocks from pupping grounds with different genetic characteristics was detected.

While populations of large coastal sharks, including sandbar sharks, have precipitously declined over the last decade because of the great increase in commercial shark exploitation, numbers of young sandbar sharks remain relatively high in the Chesapeake Bay (Musick et al., 1993). This apparent contradiction to the parent-stock recruitment relationship may be due to reduced predation on young sandbar sharks by the relative scarcity of adults of larger species (Musick et al., 1993).

Overexploitation of large sharks can have severe and long-lasting effects on shark populations because of the slow growth rate, high age at maturity, and low fecundity of large, viviparous sharks (Holden, 1974; Hoff and Musick, 1990). As catches of sharks decline in the offshore waters of the Gulf of Mexico and mid-Atlantic Bight, commercial fishermen have sought access to sharks within the nearshore coastal waters under state jurisdiction. The results of this study indicate that the young sandbar sharks of the coastal waters of Virginia are members of the same stock that has declined in number in the Gulf of Mexico and U.S. Atlantic coastal waters. Thus further exploitation of these young sandbar sharks may have impacts on the future availability of the species as far away as the Mexican

coast.

Low heterozygosities and genotypic diversities reduce the ability to test population genetic hypotheses without large sample sizes. Perhaps a technique that surveys a more variable region of the sandbar shark genome, for example direct sequencing of mtDNA or electrophoresis of microsatellite DNA (e.g. Hughes and Queller, 1993), may be more appropriate. Martin et al. (1992) have suggested that a low rate of mtDNA evolution is typical in sharks. However RFLP analysis of shortfin mako (*Isurus oxyrinchus*) mtDNA currently being performed in our laboratory indicates that low mtDNA variability is not universal in sharks; the shortfin mako has a considerably higher degree of genotypic and nucleotide sequence diversity.

Sandbar sharks in the western North Atlantic may in fact possess population structure that has not yet resulted in genetic divergence. During the Wisconsin glaciation, western Atlantic sandbar sharks may have been confined to a single refugia in the lower Gulf of Mexico and Caribbean Sea. Since the seas warmed and the range of the species extended northward, there may not have been enough time for stochastic processes to produce different frequencies of allozyme and mtDNA alleles between populations. This is possible owing to the low degree of genetic variation and slow rate of molecular evolution (Martin et al., 1992) of this species.

The characterization of the allozyme and restriction fragment patterns of the sandbar shark in the western North Atlantic can provide a baseline for evaluating the structure of the species on a cosmopolitan scale. The sandbar shark's unusual distribution pattern -- that of multiple isolated coastal populations separated by deep oceans and temperature regimes unfavorable to the species -- make it a desirable species for testing the effects of gene flow between populations that are allopatrically remote. Future analysis of sandbar shark populations may provide additional information on potential levels of genetic divergence within patchily distributed species.

CHAPTER THREE

Genetic Variation Between Atlantic and Indian Ocean Sandbar Sharks (*Carcharhinus plumbeus*) Based on RFLP Analysis of mtDNA

INTRODUCTION

The sandbar shark (*Carcharhinus plumbeus*) is a large, coastal shark that has numerous allopatric populations throughout the warm-temperate and subtropical oceans (Compagno, 1984). This species was first poorly described in the Mediterranean by Nardo (1827), and subsequently described as *C. milberti* in the western North Atlantic (Müller and Henle, 1841). With the recognition of the worldwide occurrence of the sandbar shark, Garrick (1982) synonymized sandbar sharks under the older name *C. plumbeus*.

The sandbar shark is the most important species in the US commercial longline fishery (Anon, 1993), and it also supports fisheries in Japan (Taniuchi, 1971), and Australia (Heist, pers. obs.). The species is taken in longlines off Hawaii (Wass, 1973) and in the "protective" gill nets off Natal, South Africa (Cliff et al., 1988). Significant differences in growth rates and fecundities of sandbar sharks relative to the detailed description by Springer

(1960) in the western North Atlantic were noted by Taniuchi (1971) in the South China Sea, and by Wass (1973) in Hawaii.

There have been several recent studies on the population genetics of coastal marine fishes with allopatric populations. Avise et al. (1986) determined that although American and European eels (*Anguilla sp.*) were morphologically very similar and apparently shared a common spawning area, they were genetically distinct based on mtDNA characteristics. Dodson et al. (1991) found that the distribution of mtDNA genotypes was linked to geographic region and not spawning mode in the capelin (*Mallotis villosus*). Crosetti et al. (1994) found a very close relationship between geographic propinquity and mtDNA haplotype frequency in the grey mullet (*Mugil cephalus*), and Scoles (in prep.) likewise linked mtDNA haplotype frequencies, as well as the degree of divergence between haplotypes, with geographic proximity in several species of mackerel (*Scomber*).

In this study RFLP analysis of mtDNA is used to estimate the degree of genetic divergence in sandbar sharks between two widely separated geographic regions to answer the following questions: does the degree of genetic divergence between the western North Atlantic and eastern Indian Ocean conform to complete historical isolation, or does it indicate recent gene flow? How does the degree of isolation between sandbar sharks from the two regions relate

to interspecific differences? What does the degree of inter- and intra-population variation tell us about the evolutionary history of this species? This study relies on previously published data from a comparison of population genetics within the western North Atlantic in which no significant geographic heterogeneity of mtDNA haplotypes was detected (Chapter 2).

METHODS

Sample collection.-Sandbar sharks were captured from the western North Atlantic (n=95) in the regions of the Chesapeake Bay and Gulf of Mexico and in the eastern Indian Ocean off western Australia (n=16) aboard a commercial gillnet boat out of Kalbari, West Australia (Lat 28° S; Figure 1). Heart tissue samples in the Atlantic were placed into cryovials and stored under liquid nitrogen in the field. In Australia, whole hearts were stored in plastic bags on wet ice for up to twelve hours and stored in a -20°C freezer for four to six days, then shipped to the US on dry ice. All samples were stored in a -70°C freezer until analyzed.

Mitochondrial DNA Analysis.-Mitochondrial DNA was isolated from heart tissue following the protocols outlined in chapter two and digested with the same twelve enzymes

(Appendix 1). Following electrophoresis and membrane transfer the fragments were probed with highly purified shortfin mako (*Isurus oxyrinchus*), or tiger shark (*Galeocerdo cuvier*) mtDNA nick-translated with biotin-7 dATP and visualized using the BRL BlueGene Nonradioactive Nucleic Acid Detection System.

Fragment patterns were scored for each restriction enzyme and each individual was assigned a composite genotype based on the fragment patterns for all enzymes. Each polymorphism (RFLP) could be explained by the gain or loss of one restriction site relative to another pattern. The nucleon (genotypic) diversity was calculated for each sample and for the composite of both samples following Nei (1987): Nucleotide sequence diversity was calculated following the site approach of Nei and Miller (1990). Chi-square significance of the difference in genotypic frequencies between samples was computed using the randomization protocol of Roff and Bentzen (1989). Genetic distances, diversities, and divergences were calculated using the REAP statistical analysis package (McElroy et al. 1991).

RESULTS

Sixteen sandbar sharks from the west coast of Australia were scored for the same twelve restriction enzymes used for

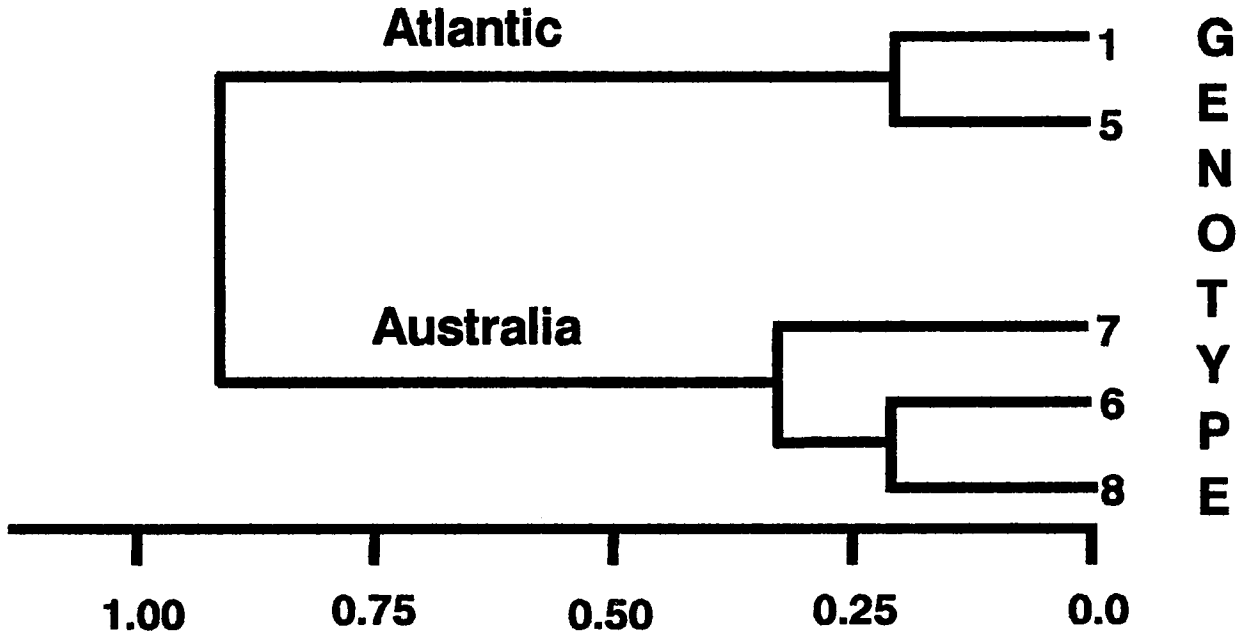
the western North Atlantic (Appendix 1; Table 3). All Australian sandbar sharks could be distinguished from Atlantic sandbar sharks by the presence of several fixed restriction site differences (Table 3, Figure 2). Three enzymes were discarded from the analysis. *Bgl* I and *Hae* II produced only a single fragment in Australian sandbar sharks (as opposed to two fragments in the Atlantic), so it could not be determined if the single restriction site was homologous in all individuals. The enzyme *Ava* II produced too many small fragments to be reliably scored in terms of homology with Atlantic samples, and was discarded from the analysis. Of the remaining nine restriction enzymes, four (*Ava* I, *Ban* I, *Dra* I, and *Hinc* II) showed fixed restriction fragment differences (Table 3). Each of these different fragment patterns could be explained by the gain or loss of one (or two in the case of *Ava* I pattern 'Y') relative to the common Atlantic genotype. The most common fragment pattern for *Hind* III was indistinguishable from a rare pattern seen in one Atlantic individual, while a rare pattern seen in one Australian individual was indistinguishable from the common Atlantic type (Appendix I, Table 3). The Australian sandbar sharks exhibited a much higher level of genotypic and nucleotide sequence diversity than the Atlantic sandbar sharks (Table 3) based on nine enzymes, however the diversity in the western North ATlantic based on twelve enzymes (chapter 2), was only slightly

Table 3. Mitochondrial restriction patterns observed, genotypes designated, number of genotypes observed in the sandbar shark (*Carcharhinus plumbeus*) from the northwest Atlantic (US) and Eastern Indian Oceans (Australia). Enzymes scored (left to right) are *Ava* I, *Ban* I, *Bcl* I, *Dra* I, *Hinc* II, *Hind* III, *Nru* I, *Sca* I, *Xho* I.

Genotype	Pattern	US	AUSTRALIA
1	A A A A A A A A A	94	0
5	A A A A A B A A A	1	0
6	X X A X X B A A A	0	14
7	X X A X X A A A A	0	1
8	Y X A X X B A A A	0	1
Total		95	16
Haplotype Diversity		0.02	0.23
Nuleotide Sequence Diversity		0.004%	0.050%

Corrected Nucleotide Sequence Divergence = 1.03%

Figure 2. UPGMA clustering of nsd of sandbar shark (*Carcharhinus plumbeus*) mtDNA genotypes from the western North Atlantic and eastern Indian Oceans.



Mean nucleotide sequence divergence = 1.02%

lower. The corrected nucleotide sequence divergence between American and Australian sandbar sharks was 1.03%.

DISCUSSION

Sandbar sharks in the eastern Indian Ocean had about a tenfold increase in intraspecific mtDNA variation relative to western North Atlantic sandbar sharks (haplotype diversity in the Atlantic/Indian = 0.02/0.23, nucleotide sequence diversity in the Atlantic/Indian = 0.004%/0.050%). The Indian Ocean sample was smaller, and thus subject to a greater degree of sampling error, and based on the higher haplotype and nucleotide sequence diversities based on twelve enzymes in the western North Atlantic, this difference in diversity appears to be a sampling artefact.

If intrapopulation diversity is higher in the eastern Indian Ocean, this result could be explained by a much greater effective population size in the Indian Ocean. It is not known if the sandbar sharks of the west coast of Australia are contiguous with those off the Asian Pacific mainland and the northeast coast of Australia (Compagno, 1984). If the sandbar sharks along the west coast of Australia are an isolated population then it is unlikely that they have a population size much larger than that of the western North Atlantic. Another explanation for the difference in variation is that the sandbar sharks of the

western North Atlantic are a recent founder population, perhaps established by migration from the coast of western Africa as hypothesized by Springer (1960). If Springer's hypothesis is correct, then sandbar sharks from the Mediterranean and western Africa should have similar genotypes but a higher genetic diversity than those from the western North Atlantic. Other cases of regional differences in genetic variation have been reported. Graves and McDowell (1994b) found that blue marlin (*Makaira nigricans*) exhibited higher mtDNA diversity in the Atlantic than in the Pacific Ocean, Graves et al. (1992) found that bluefish (*Pomatomus saltatrix*) also had a higher mtDNA diversity in the western North Atlantic than in the Pacific waters off Australia, and Crosetti et al. (1994) reported that haplotype and nucleotide sequence diversity varied markedly over the cosmopolitan range of the grey mullet (*Mugil cephalus*).

If a constant rate of genetic change can be assumed, it is possible to estimate the age of evolutionary events based on nucleotide sequence divergence. Using the standard vertebrate molecular clock in primates and ungulates of 2% nucleotide substitutions per million years (Brown et al., 1979; Wilson et al. 1985) the amount of divergence between western North Atlantic and Australian sandbar sharks amounts to about 0.5 million years of divergence, while using the "slower" molecular clock of Martin et al. (1992), this

divergence amounts to approximately three-four million years of separation. At any rate the populations are completely isolated and have been for some time.

Although this amount of interpopulation divergence is large relative to the intrapopulation diversity, it is small relative to interspecific differences. Genetic divergence between the sandbar shark and the dusky shark (*C. obscurus*) could not be estimated from RFLP data because so few of the fragments produced were comigratory that no reliable homologies could be assigned (Heist, unpublished data). This result is somewhat contradictory to the conclusions of Martin (1993) based on direct sequence analysis of the cytochrome *b* gene. Martin (1993) reported percent divergences of 1.2% (*C. plumbeus* vs. *C. altimus*) and 1.9% (*C. longimanus* vs. *C. obscurus*) between closely related species of *Carcharhinus* based on cytochrome *b* direct sequence data. It appears that a molecular clock based solely on cytochrome *b* runs slower than one revealed by RFLP analysis of the entire mitochondrial molecule.

The genus *Carcharhinus* currently comprises 36 nominal species. Many of them, like the sandbar shark, have numerous allopatric populations. Several nominal species have been synonymized over the last few decades and one "cryptic" species was recently identified on the basis of genetic (allozyme) data (Lavery and Shaklee, 1989). Most temperate and subtropical coastlines support several species

of *Carcharhinus*, often with greatly overlapping prey preferences and foraging locations. In the field work for this study four coastal species of *Carcharhinus* (*C. brevipinna*, *C. limbatus*, *C. plumbeus*, and *C. obscurus*) were collected in both Australia and Virginia.

The occurrence of fixed allelic differences of mitochondrial haplotypes is similar to the findings of Graves et al. (1992) for the bluefish (*Pomatomus saltatrix*) between Australia and the western North Atlantic. In that study bluefish had fixed allelic differences in mtDNA revealed by three of nine restriction enzymes. Perhaps future will reveal whether there is a strong relationship between geographic proximity of other populations of sandbar sharks and the degree of genetic divergence as in the grey mullet (Crosetti et al., 1994), or if other species of *Carcharhinus* share the same patterns and magnitudes of genetic divergence.

CHAPTER FOUR

Cosmopolitan population genetics of the shortfin mako (*Isurus oxyrinchus*)

The shortfin mako (*Isurus oxyrinchus*) is a large (maximum length 4 m), active, pelagic shark that inhabits the temperate and subtropical oceans of the world. This species has traditionally been discarded as bycatch in the swordfish longline fishery. However, as the catches of swordfish decline, and the public acceptance of mako flesh increases, more makos are being harvested commercially (Casey and Kohler, 1992). The shortfin mako's reputation for jumping and making long runs when hooked has made it a much pursued game fish, and has led to the establishment of mako fishing tournaments throughout the world (Casey and Kohler, 1992; Holts and Bedford, 1993; Pepperell, 1992; Stevens, 1992). Like other members of the Lamnidae, the shortfin mako possesses a rete mirabile heat exchanger that maintains the visceral temperature well above the ambient water temperature (Carey and Teal, 1969; Carey et al. 1981). Because of their great range and vagility, Compagno (1984) referred to this species as the "peregrine falcon of the shark world".

While both sexes of shortfin mako grow at approximately

the same rate, females tend to mature later and grow larger (Pratt and Casey, 1983). Reproduction is ovoviviparous with females producing four to sixteen large (70 cm total length) oviphagous pups (Stevens, 1983). It is unknown where makos mate, and there are only incidental records of pregnant and post-partem females, due in part because of the difficulties in catching such large, powerful animals.

Despite the commercial and recreational interest in this species, little is known about the movements of individuals. Tag/recapture studies have documented movements of greater than 1000 km, with individuals tagged in US Atlantic coastal waters recaptured as far away as the Azores and Venezuela (Casey and Kohler, 1992). Compagno (1984) reported that the mako tends to move towards higher latitudes during warmer months, and Casey and Kohler (1992) hypothesized that some individuals may make a complete circuit of the North Atlantic from the mid-Atlantic coast in the spring, to the North central Atlantic in the summer, and on through the "sargasso sea" to the Caribbean and Gulf of Mexico in the winter. This route would correspond well with the abundance of food fish and preferred water temperatures.

Movements of makos may be to a large part influenced by water temperature. Direct telemetry data (Carey et al. 1981; Holts and Bedford, 1993), as well as inference from longline catch data (Casey and Kohler, 1992), indicate that

the preferred temperatures of shortfin makos range from 17 to 22 °C, with individuals making only brief forays into cold waters below. Carey and Scharold (1990) reported on a single, large individual that went as deep as 480 m in the well-mixed waters of the Gulf Stream, while three juveniles (total length 1.7-1.8 m) telemetered in the stratified waters of the California Bight never descended deeper than 40 m, remaining in the warmer mixed waters above (Holts and Bedford, 1993). While off the US east coast in the spring, the mako's northward migration parallels that of the bluefish (*Pomatomus saltatrix*), and at these times this prey species comprises the majority of stomach content volume (Stillwell and Kohler, 1982).

There have been numerous studies on the population genetics of pelagic nektonic fishes. Studies of the skipjack tuna (*Katsuwonis pelamis;* Graves et al. 1984) and the yellowfin tuna (*Thunnus albacares;* Scoles and Graves, 1993) have detected no heterogeneity in allele frequencies between ocean basins. However studies of several species of istiophorids have detected stock structure both within (Graves and McDowell, 1994a) and between (Finnerty and Block, 1992; Graves and McDowell, 1994b) ocean basins. The purpose of this study is to test the hypothesis that the shortfin mako shares a single cosmopolitan homogeneous gene pool, and if this hypothesis is rejected, to determine where barriers to gene flow may occur. Because of the maternal

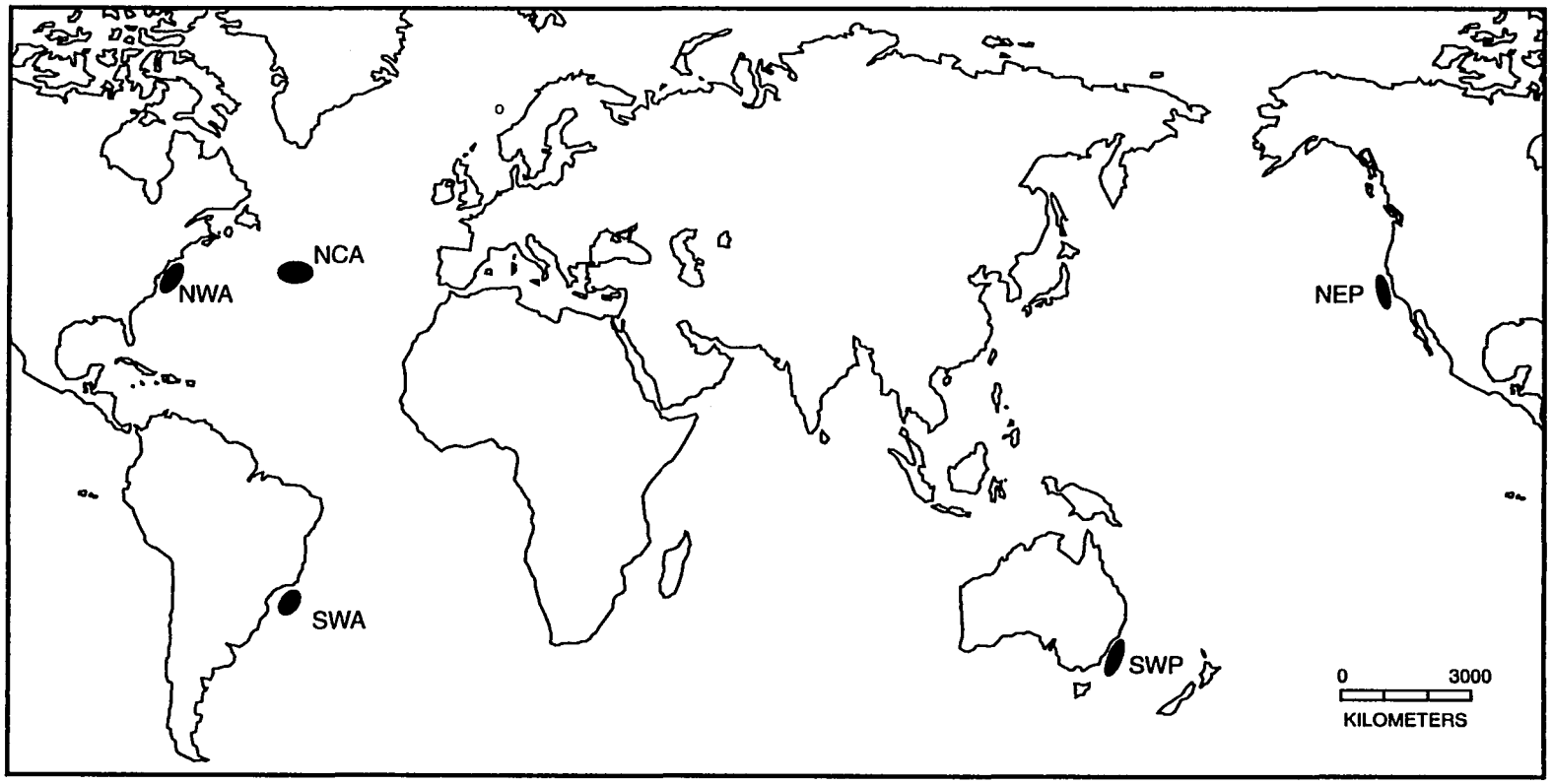
inheritance of mtDNA it is likely that the distribution of genotypes will be determined by the movements of the largest individuals of the species.

Materials and Methods

Sample collection.-Shortfin makos were collected from five distant locations (Figure 2) with the aid of numerous scientists. Heart tissue was collected at sportfishing tournaments in the mid-Atlantic Bight from New York and New Jersey (n=18), supplemented by three makos from VIMS research longline cruises. A central Atlantic sample (n=24) was collected aboard a Spanish swordfish longline vessel northwest of the Azores (Lat 41-43.5° N, Long 36.5-41° W). Southwest Atlantic samples (n=17) were collected from Brazilian longline vessels out of Santos, Brazil. Southwest Pacific makos (n=22) were collected from sportfish tournaments in New South Wales, Australia. Northeast Pacific makos (n=30) were collected at sportfishing tournaments in the southern California bight and Channel Islands. All samples were stored until analysed at -70°C.

Mitochondrial DNA RFLP Analysis.-Mitochondrial DNA was isolated from heart tissue, digested with restriction enzymes, and electrophoresed following the protocols

Figure 3. Sample locations for the shortfin mako (*Isurus oxyrinchus*). Sites are as follow: NWA = western North Atlantic (n = 21), NCA = central North Atlantic (n = 24), SWA = western South Atlantic (n=17), SWP = Southwest Pacific (n = 22), and NEP = eastern North Pacific (n=30).


NCA
NWA
NEP
SWA
SWP
0
3000
KILOMETERS

outlined in chapter two. Following membrane transfer the fragments were probed with highly purified shortfin mako, porbeagle (*Lamna nassus*), or tiger shark (*Galeocerdo cuvier*) mtDNA nick-translated with biotin-7 dATP and visualized using the BRL BlueGene Nonradioactive Nucleic Acid Detection System.

Fragment patterns were scored for each restriction enzyme and each individual was assigned a composite genotype based on the fragment patterns for all enzymes. Each polymorphism (RFLP) could be explained by the gain or loss of one restriction site relative to the common pattern (Figure 4). The nucleon (genotypic) diversity was calculated for each sample and for the composite of both samples following Nei (1987). Nucleotide sequence diversity was calculated following the site approach of Nei and Miller (1990). Chi-square significance of the difference in genotypic frequencies between samples was computed using the randomization protocol of Roff and Bentzen (1989). Genetic distances, diversities, and divergences were calculated using the REAP statistical analysis package (McElroy et al., 1991).

RESULTS

A considerable amount of genetic variation was detected in the shortfin mako compared to previous studies on sharks (chapter two; Martin, 1993). A survey of nine restriction enzymes detected 24 genotypes in 114 individuals (Appendix II, Figure 4) for an overall haplotype diversity of 0.815 (Table 4). All nine enzymes produced multiple fragment patterns. The mean nucleotide sequence diversity was 0.362%. The nine restriction enzymes surveyed an average of 221 base pairs or approximately 1.34% of the 16.5 kb mitochondrial genome.

Significant spatial partitioning of mtDNA haplotypes was detected among shortfin mako samples. The two North Atlantic samples had the lowest genotypic and nucleotide sequence diversities, wheras the two Pacific samples had the highest diversities (Table 4). The overall Roff-Bentzen chi square probability for the null hypothesis that all five samples could have been drawn from a single gene pool was <0.001. Haplotypes did not seem to be confined to particular regions (Figure 4), the three most common genotypes were found in all five samples.

Because tag/recapture data indicated movement of makos between the western North Atlantic and central North

Atlantic sample sites (Casey and Kohler, 1992), an initial pairwise comparison was done to determine the suitability of pooling these samples (Table 5). Out of 1000 randomizations, 184 had the same or higher contingency chi-square values ($p = 0.184$), so the null hypothesis of a single North Atlantic gene pool was not rejected and these samples were combined. Pairwise comparisons were performed between all pairs of the resultant four samples (Table 6), and only those comparisons between the pooled North Atlantic sample and the other three locations (Brazil, Australia, California) were significant ($p < 0.001$). These comparisons were significant even when the α level was decreased in a sequential Bonferroni procedure to adjust for multiple testing (Rice, 1989). Comparisons between the Brazil, Australia, and California samples were all nonsignificant ($p = 0.136$ to 0.406; Table 6).

The nucleotide sequence divergences between the North Atlantic and the other three locations ranged from 0.034 to 0.062% (Table 6), indicating that approximately 9-17% of the genetic divergence between individuals between the regions could be explained by between region differences. The nucleotide sequence divergences between the Brazil, Australia, and California samples ranged from -0.009 to +0.002%, indicating that practically none of the genetic variation between individuals between these regions could be explained by between region differences. Pairs of

Table 4. Mitochondrial DNA restriction fragment patterns found in the shortfin mako (*Isurus oxyrinchus*) and their geographic distributions. Restriction enzymes (in order) are *Ava* I, *Bcl* I, *Bgl* I, *Bst*E II, *Dra* I, *Hae* II, *Hind* III, *Hpa* I, *Stu* I.

Restriction Pattern	N. west Atlantic	N. central Atlantic	S. west Atlantic	S. west Pacific	N. east Pacific	Total
AAAAAAAAA	0	0	0	3	4	7
AAAAAAABA	2	6	5	4	10	27
AAAAAAABB	0	0	1	0	0	1
AAAAAAADA	0	0	1	0	0	1
AAAAAABBA	0	0	0	1	0	1
AAAACAABA	0	0	1	0	0	1
AAABAAAAA	15	13	2	5	3	38
AAABAAAAB	1	0	0	0	0	1
AAABAAABA	2	2	3	1	3	11
AAABAAACA	1	0	0	0	0	1
AAABAAADA	0	0	0	1	0	1
AAABABAAA	0	0	0	0	3	3
AAABBAAAA	0	0	0	0	1	1
AABBAAAAA	0	3	0	1	0	4
AAACAAADA	0	0	0	1	0	1
ABAAAAABA	0	0	1	2	1	4
ABABAAAAA	0	0	0	2	0	2
ABABABAAA	0	0	0	1	0	1
ACAAAAABA	0	0	1	0	2	3
BAABAAAAA	0	0	0	0	1	1
BAABAAABA	0	0	0	0	1	1
CAABAAABA	0	0	0	0	1	1
DAABAAAAA	0	0	1	0	0	1
EAAAAAAAA	0	0	1	0	0	1
TOTAL	21	24	17	22	30	114

Table 5. Comparison between northwestern and northcentral Atlantic mtDNA haplotypes in the shortfin mako (*Isurus oxyrinchus*).

GENOTYPE	Northwest Atlantic	Northcentral Atlantic	TOTAL
2	2	6	8
7	15	13	28
8	1	0	1
9	2	2	4
10	1	0	1
14	0	3	3
TOTAL	21	24	45
Haplotype Diversity	0.491	0.649	
% Nucleotide Sequence Diversity	0.160	0.251	

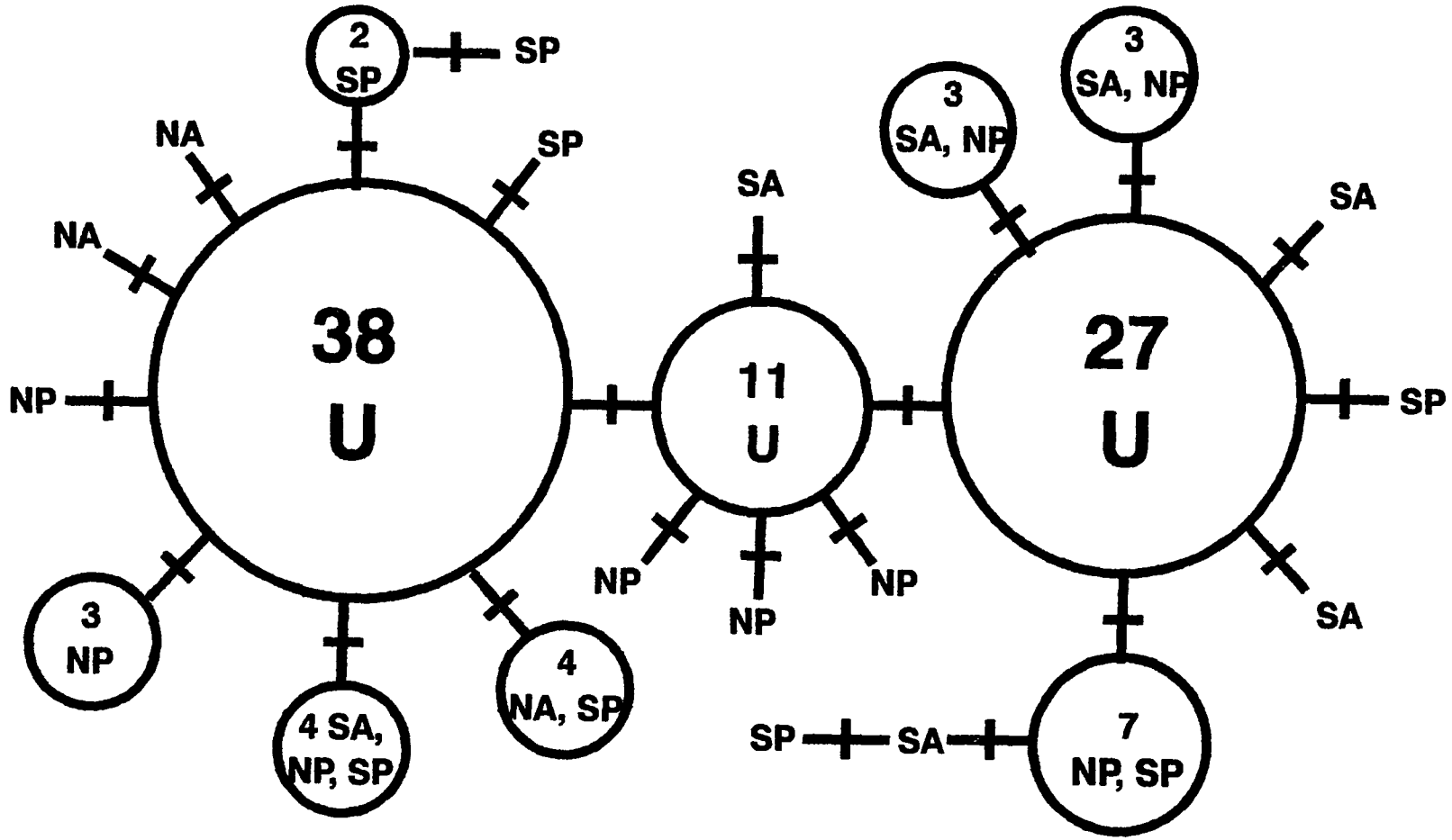
Roff and Bentzen chi-square probability = 0.184

Corrected percent nucleotide sequence divergence = 0.006

Table 6. Roff-Bentzen probability of genetic homogeneity (above diagonal) and corrected nucleotide sequence divergence (below diagonal) for the shortfin mako (*Isurus oxyrinchus*).

	North Atlantic	Southwest Atlantic (Brazil)	Southwest Pacific (Australia)	Northeast Pacific (California)
North Atlantic	*******	<0.001	<0.001	<0.001
Southwest Atlantic (Brazil)	0.062	*******	0.200	0.406
Southwest Pacific (Australia)	0.034	-0.001	*******	0.136
Northeast Pacific (California)	0.037	-0.009	0.002	*******

Figure 4. Relationships among mtDNA genotypes in the shortfin mako (*Isurus oxyrinchus*). Numbers in circles are the total number of individuals with a particular genotype, areas of circles are proportional to the number of individuals possessing that genotype. Genotypes possessed by only one individual do not have numbers or circles. Geographic locations are as follows: NA = North Atlantic, SA = South Atlantic, NP = North Pacific, SP = South Pacific, U = ubiquitous.


38
U
11
U
27
U
2
SP
SP
SP
NA
NA
NP
3
NP
4 SA,
NP, SP
4
NA, SP
SA
NP
NP
NP
3
SA, NP
3
SA, NP
SA
SA
SP
SA
SP
7
NP, SP

individuals within these samples were on average as divergent as pairs of individuals between these regions.

DISCUSSION

The shortfin mako exhibits a great degree of intraspecific genetic variation, and significant partitioning of genotypes between the North Atlantic and other regions. Makos exhibit less variation in the North Atlantic than in the combined Indo-Pacific and South Atlantic Oceans. While there may be separate fishery stocks within the Indo-Pacific and South Atlantic, our results indicated that there has historically been enough exchange between these areas to prevent the accumulation of genetic divergence between regions. Thus in an evolutionary sense we have detected only two populations.

Based on the geographic extent of suitable habitat in the North Atlantic and the combined South Atlantic and Pacific, it is reasonable to assume that there are more makos in the latter region than in the North Atlantic. The lower degree of genetic variation within the North Atlantic may be due to the smaller effective population size in the North Atlantic.

The difference in trans-equatorial migration between the Atlantic and Pacific can perhaps be explained by the

thermal preference of the species. Direct telemetry data in the North Pacific (Holts and Bedford, 1993) as well as temperature/
occurrence data inferred from longline data in the Atlantic (Hoey, 1993, as cited in Casey and Kohler, 1992) show that the shortfin mako prefers waters ranging from 14° to 22° C. While the Gulf Stream maintains a thick (>500 m) mixed stratum throughout this temperature range, the surface waters of the equatorial Atlantic are always above 24° C, and there is a permanent steep thermocline that rapidly declines to below 5° C (Pickard and Emery, 1982). Therefore the vertical extent of the mako's preferred temperature range is limited. Unlike the static thermal structure of the equatorial Atlantic, the equatorial Pacific undergoes considerable seasonal variation in temperature (Pickard and Emery, 1982), and perhaps the thermal disturbance caused by periodic el niño events also facilitates trans-equatorial movements within the Pacific.

The lack of significant genetic structure detected in the shortfin mako between Brazil, Australia, and California does not necessarily mean that these widespread areas support only a single, panmictic stock of shortfin mako. The absolute number of migrants between regions that is necessary to prevent genetic drift from producing significantly different allele frequencies is very small, on the order of one to ten individuals per generation

(Allendorf and Phelps, 1981). Fisheries-relevant stocks may exist in the absence of any detectable genetic divergence. Only a large international tag/recapture study can address movements on this scale.

The shortfin mako exhibits considerable morphological variation. When Garrick (1967) revised the genus *Isurus* there were as many as twelve nominal species, and authoritative accounts varied from two to four species. Garrick's revision, which acknowledged considerable morphological variation within the shortfin mako, collapsed most nominal species into *Isurus oxyrinchus*, and described a new species, the longfin mako (*Isurus paucus*). Recently Moreno and Morón (1992) described several individual mako sharks from the Azores with atypical pigment patterns and suggested that these individuals represented a special form endemic to the Azores.

The results of our study do not support the presence of additional "cryptic" species of makos, although such individuals could easily have been missed by the sampling. All fragment patterns detected could be explained by the gain or loss of a single restriction site relative to another fragment pattern; all genotypes were no more than one observed mutational step away from another genotype (Figure 4).

Eight individuals from the Brazilian sample had fragment patterns vastly different from the common shortfin

mako pattern. However these individuals shared fragment patterns with North Atlantic porbeagle sharks (*Lamna nassus*), and it was concluded that the true species of these samples were mistaken in the field, and they were thus excluded from the survey. Based on the results of this study, the shortfin mako appears to be a single evolutionary species with two partially isolated genetic populations, one in the North Atlantic, and one ranging throughout the rest of the world's temperate and sub-tropical oceans.

The results of this study are somewhat in contrast to studies of another large pelagic fish, the blue marlin (*Makaira nigricans*). Based on both direct sequencing of the mitochondrial cytochrome *b* gene (Finnerty and Block, 1992) and RFLP analysis of mtDNA (Graves and McDowell, 1994b), unique genotypes were detected in the Atlantic that were apparently absent in the Pacific. The degree of intrapopulation variation was also higher in the Atlantic than the Pacific, with Pacific genotypes occurring within the Atlantic, but unique Atlantic genotypes observed to be several mutational steps away from any genotypes observed in the Pacific. Graves and McDowell (1994b) attributed this result to a complete isolation of blue marlin populations at some time in the past. Our study does not detect any historical isolation of mako stocks as all observed genotypes were no more than one observed mutational step away from another, and there appeared to be no significant

regional partitioning of mtDNA haplotypes (Figure 4).

The significant genetic structure between the North and South Atlantic in the shortfin mako has important management implications. If the shortfin mako is overfished in the North Atlantic, replenishment by migration from other regions will be very slow. Because the two non-divergent samples collected in the North Atlantic were harvested by fisheries from Europe and North America, and because these same makos are harvested by longliners from as far away as Japan (Casey and Kohler, 1992), management efforts will be complicated by the difficulties of international agreement.

CHAPTER FIVE

Population Genetics of the Atlantic Sharpnose Shark (*Rhizoprionodon terraenovae*).

INTRODUCTION

The Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) is a small (maximum length 110 cm) coastal shark that inhabits the east coast of North America from New Brunswick, Canada to Yucatan, Mexico. The species is very abundant in parts of its range, and supports a commercial fishery in Mexico (Bonfil et al., 1993) as well as a recreational fishery off Texas (Parrack, 1990). In the mid-Atlantic bight the Atlantic sharpnose shark is second only to the sandbar shark (*Carcharhinus plumbeus*) in longline catches (Musick et al., 1993).

The Atlantic sharpnose shark has a much faster replacement rate than many larger species of sharks, reaching sexual maturity at two to three years of age, and having up to seven young every year thereafter (Parsons, 1983). The species pups in both the South Atlantic Bight and in the Gulf of Mexico (Compagno, 1984).

Currently catches of small coastal species (predominantly the Atlantic sharpnose shark) are not regulated because catch rates are considered to be at or below maximum sustainable yield (Anon, 1993; Parsons, 1990), and because the relatively rapid replacement rate for this species. However if the commercial quotas for large coastal species become more restricted, small coastal species may become targeted, and it will become important to know if small coastal sharks can be managed as discrete stocks between the southeastern Atlantic and Gulf of Mexico.

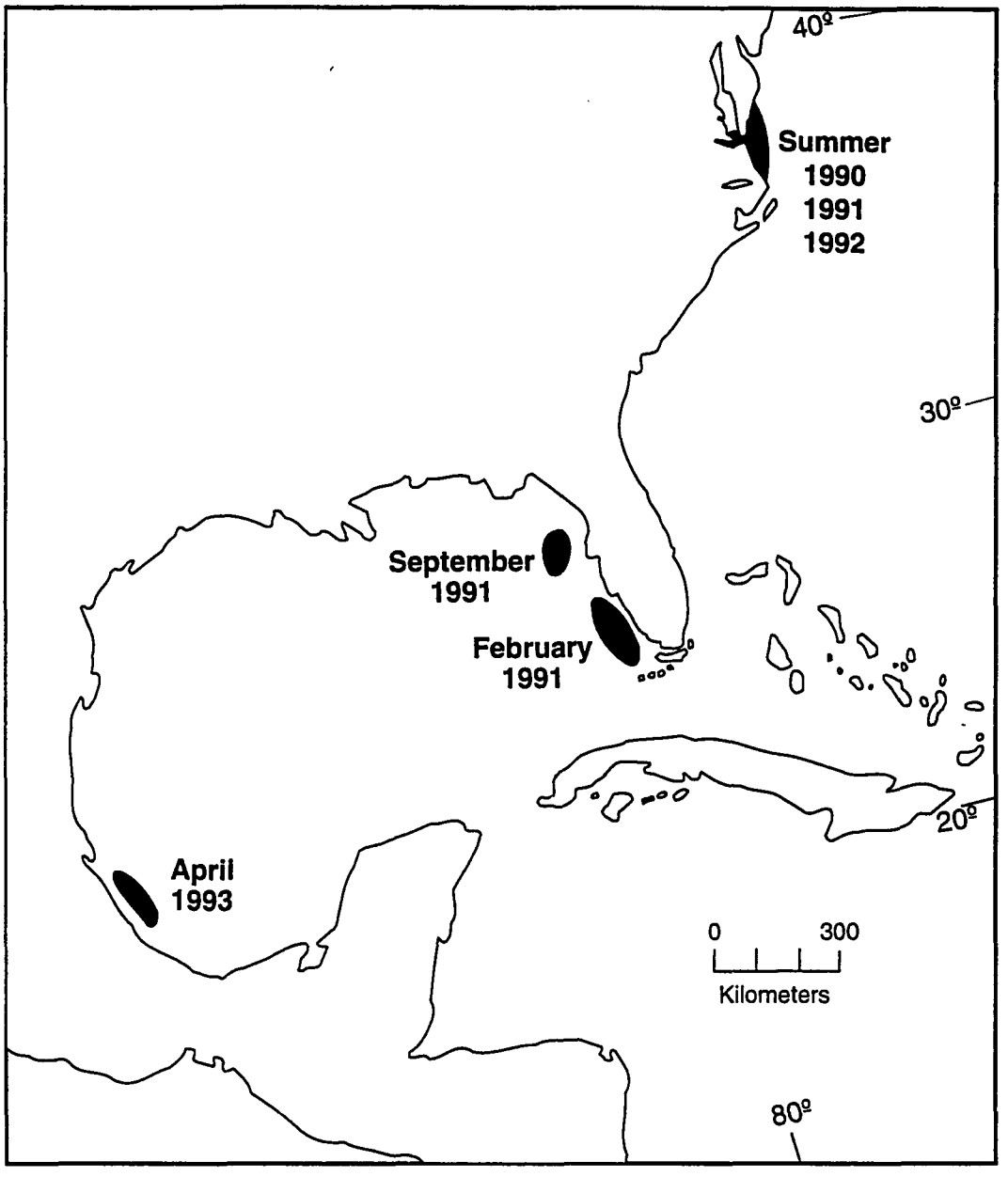
METHODS

Sample collection.-Atlantic sharpnose sharks were collected in the mid-Atlantic Bight (n=23) with research longlines as part of the ongoing shark research program of the Virginia Institute of Marine Science (VIMS), from the recreational fishery of southern Texas (n=21), and from the commercial fishery off Veracruz, Mexico (n=8). Heart tissue samples in the Atlantic were placed into cryovials and stored under liquid nitrogen in the field. In Texas and Mexico, whole hearts were collected on wet ice and stored frozen until shipped to Virginia. All samples were stored in a -70 °C freezer until analyzed.

Mitochondrial DNA Analysis.-Mitochondrial DNA was isolated from heart tissue following the protocols outlined in chapter two and digested with a panel of ten restriction enzymes (Table 7). Following electrophoresis and membrane transfer the fragments were probed with highly purified tiger shark (*Galeocerdo cuvier*) mtDNA nick-translated with biotin-7 dATP and visualized using the BRL BlueGene Nonradioactive Nucleic Acid Detection System.

Fragment patterns were scored for each restriction enzyme and each individual was assigned a composite genotype based on the fragment patterns for all enzymes. Each polymorphism (RFLP) could be explained by the gain or loss of one restriction site relative to another pattern. The nucleon (genotypic) diversity was calculated for each sample and for the composite of both samples following Nei (1987). Nucleotide sequence diversity was calculated following the site approach of Nei and Miller (1990). Chi-square significance of the difference in genotypic frequencies between samples was computed using the randomization protocol of Roff and Bentzen (1989). Genetic distances, diversities, and divergences were calculated using the REAP statistical analysis package (McElroy et al., 1991).

Figure 5. Locations and dates for collection of the sharpnose shark (*Rhizoprionodon terraenovae*).


40º
Summer
1990
1991
1992
30º
September
1991
February
1991
20º
April
1993
0
300
Kilometers
80º

RESULTS

Genetic diversity in the Atlantic sharpnose shark is higher than in the sandbar shark, but lower than in the shortfin mako. A total of seven haplotypes were detected with an overall nucleotide sequence diversity of 0.18% (Table 7). The most common haplotype was found with similar frequencies in each sample, and four rarer haplotypes also occurred in each sample. Three unique haplotypes were detected, one from each sample location. Roff and Bentzen (1989) chi-square analysis produced a probability value of 0.694, suggesting that all three samples could have been drawn from a single gene pool. The between sample corrected nucleotide sequence divergences were all less than 0.01%.

DISCUSSION

Based on the results of this study we could not reject the null hypothesis that there is a single contiguous Atlantic sharpnose shark stock from Virginia to Veracruz, Mexico. While it is possible that migration between the Gulf of Mexico and southeastern US Atlantic coast is limited enough to allow for the maintenance of separate fishery stocks, this hypothesis can not be tested by our data. If

Table 7. Mitochondrial DNA restriction fragment patterns found in the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) and their geographic distribution. Restriction enzymes (in order) are *Ava* I, *Ava* II, *Ban* I, *Bcl* I, *Bst*E II, *Dra* I, *Hind* III, *Hpa* I, *Sca* I, *Xho* I.

GENOTYPE	VIRGINIA	TEXAS	MEXICO	TOTAL
AAAAAAAAAA	14	14	4	32
AAAAAAABAA	1	0	0	1
AAAAAAACAA	5	4	1	10
AAAAAAADAA	2	1	1	4
AAAAAABAAA	0	1	0	1
AAABAAAAAA	1	1	1	3
BAAAAAAAAA	0	0	1	1
TOTAL	23	21	8	52
HAPLOTYPE DIVERSITY	0.597	0.538	0.786	0.640
% NUCLEOTIDE SEQUENCE DIVERSITY	0.163	0.136	0.243	0.181

separate fishery stocks exist, either gene flow between the regions has been extensive enough to homogenize allele frequencies, or the separation is recent enough that an equilibrium divergence level has not been achieved.

It is likely that this species -- in contrast to the other two species in this study -- does consist of a single panmictic population. While tagging data exists for many large species of sharks, data is scant for this small species. Only tagging can provide information on the magnitude of migration between regions in this species.

CHAPTER SIX

GENERAL DISCUSSION

The three species examined in this study varied greatly in intraspecific and interpopulation genetic variation. Genetic variation was lowest in the sandbar shark and highest in the shortfin mako, with the Atlantic sharpnose shark intermediate in both haplotype and nucleotide sequence diversity. Although the intrapopulation variation was extremely low in the sandbar shark, the mean interpopulation nucleotide sequence divergence between the western North Atlantic and the eastern Indian Ocean was greater than that between any locations sampled in the shortfin mako.

Based on a relatively small degree of genetic divergence in mtDNA sequence differences between genera relative to divergence times inferred from the fossil record, Martin et al. (1992) suggested that the rate of mtDNA evolution in sharks was slow relative to mammals. This theme was reiterated by Martin and Palumbi (1993) in a study that contained a small number of intraspecific comparisons, including four sandbar sharks. Martin and Palumbi (1993) stated that "[t]hese preliminary surveys of intraspecific sequence variation suggest that the gene trees

and species trees for sharks are equivalent, and that there are likely to be few, if any, amino acid differences between conspecific individuals". Further Martin (1993) suggested that nucleotide sequence variation alone should be sufficient to distinguish between species, and that RFLP analysis of a small (rapidly evolving) amplified portion of the mitochondrial molecule would be an economical means of acquiring the same information. The results of this study demonstrate considerably more intraspecific variation than was anticipated from these previous studies. It may be difficult to acquire diagnostic RFLP patterns for some species without sampling over a wide geographic range (e.g. sandbar shark), or sampling a large number of individuals (e.g. shortfin mako).

What might account for the differences in intraspecific variation between these species? The amount of variation resident in the mitochondrial DNA of a population is an equilibrium between mutation and extinction of rare alleles (Nei, 1987). Because mitochondrial DNA is maternally inherited, a random pair of individuals can be expected to have inherited a mitochondrial genome from a common female ancestor after approximately $2N_{f(e)}$ generations (Nei, 1987), where $N_{f(e)}$ = the effective female population size. Thus the amount of mtDNA variation present in a species at equilibrium is a function of the population size, the mitochondrial mutation (i.e. mtDNA "clock") rate, and the

generation length. Bowen and Avise (1990) explained the extremely high rate of genetic variation (mean intrapopulation nsd = 3.2%) within the Atlantic menhaden (*Brevoortia tyrannus*) by the astronomical population size ($>10^{12}$ individuals) of this planktivorous species. While the estimated $N_{f(e)}$ was considerably less than this, Bowen and Avise (1990) attributed this to a severe fishery decimation in previous decades and noted that $N_{f(e)}$ typically underestimate census estimates. Likewise Dodson et al. (1991) attributed high intraspecific mtDNA to large population size in the capelin (*Mallotus villosus*), another very abundant planktivore.

Sandbar sharks exhibit a much lower degree of mtDNA variation than shortfin makos and Atlantic sharpnose sharks. One major life-history difference between these species is generation length. While female shortfin makos reach maturity in about six years (Pratt and Casey, 1983), and Atlantic sharpnose sharks at two to three years (Parsons, 1983), the sandbar shark does not reach maturity until an age of at least thirteen, and some individuals may not be mature until an age of thirty (Casey and Natansen, 1992). This means that the rate of extinction of rare genotypes due to genetic drift should be very slow in sandbar sharks.

Another explanation for low genetic variation in the presence of apparently high population levels is that the populations went through recent bottlenecks, or that they

were established by a small founder populations. In light of the distribution of the sandbar shark, as well as that of other species of *Carcharhinus*, it seems reasonable that many populations of Carcharhinids are the products of founder effects.

If shark stocks are to be conserved, it is critical that information on genetic stock structure be acquired. Analyses of genetic stock structure can be used to infer movements of individuals within and between the jurisdictional areas of regulating agencies, however there can still be fishery-relevant structure in the absence of genetic divergence. While genetic studies do not resolve movements as precisely as tagging studies, they can reveal mating structure in the presence of temporal mixing of biological stocks (Karl et al, 1992), or of historical zoogeographic patterns that result in current distributions (Dodson et al, 1991; Taylor and Bentzen, 1993). Limitations of tagging studies include difficulty of spreading effort of tagging and recapture evenly throughout the range of a species, and acquiring international cooperation with a tagging program. In genetic studies effort can be entirely controlled by the researcher. Results of tagging studies of fishes often imply one-way movement of stocks as an artifact of tagging and harvest being concentrated in specific regions of the range of a species. Perhaps a better overall approach to studying the population structure of a species

is to do the genetic analysis first, and then plan a tagging strategy to reveal movements that are beyond the level of resolution of genetic divergence.

Compared to the number of population genetic studies performed on other commercially important fishes, the amount of effort applied to understanding populations of sharks, or even identifying the species present in a location, is sparse. This is unfortunate because the life history characteristics of sharks make them especially vulnerable to overfishing in the absence of informed management. Several nations have sustained long term fisheries for sharks (e.g. Mexico; Applegate et al., 1993) without overfishing, in part because the relatively limited technology and low effort allowed for enough escapement that stocks were not significantly threatened. As stocks of traditional finfish decline (e.g. swordfish and tuna), nations with more efficient technology (US and Japan) are targeting sharks. The effect of increased international fishing for sharks with state of the art technology will be a decline in the traditional artisanal fisheries, bringing economic hardship to already impoverished regions. More studies of population genetics in sharks are needed to provide the information necessary to prevent this unfortunate scenario.

Appendix I. Estimated size in kilobase pairs (kb) of mtDNA restriction fragments for various restriction enzymes in the sandbar shark (*Carcharhinus plumbeus*). Letter in parentheses refers to fragment pattern designation, letters 'X' and 'Y' refer to those patterns found only in Australia.

	Ava I			*Ava* II		
(A)	(X)	(Y)	(A)	(B)	(C)	(D)
5.48	5.48	5.48	6.05	6.05	6.05	6.05
5.27	4.67	4.67	2.20	2.20	2.20	2.20
2.44	2.44	3.02	2.20	1.86	2.20	2.20
1.38	1.38	1.38	1.60	1.60	1.60	1.60
1.13	1.13	1.13	1.09	1.09	1.55	1.09
0.95	0.95	0.95	1.09	1.09	1.09	1.00
	0.58		0.87	0.87	0.87	0.87
16.65	16.63	16.63	0.77	0.77	0.77	0.77
			0.63	0.63	0.63	0.63
			0.30	0.30		0.30
			0.20	0.30	16.96	0.20
			17.00	0.20		16.91
				16.96		

Appendix I (continued)

Ban I		*Bcl* I	*Bgl* I	*Dra* I	
(A)	(X)	(A)	(A)	(A)	(X)
7.10	10.17	6.75	12.00	5.65	10.46
5.65	7.10	4.18	4.71	4.81	2.32
4.52		2.24		2.32	1.93
	17.27	1.84	16.71	1.93	1.52
17.27		1.30		1.52	
		0.95			16.23
				16.23	
		17.26			

Hae II	*Hinc* II	*Hind* III		*Nru* I	*Sca* I	Xho I
(A)	(A)	(A)	(B)	(A)	(A)	
9.50	3.76	4.69	4.69	12.00	9.50	9.00
7.00	3.42	3.55	3.55	4.50	6.07	5.31
	3.23	3.28	3.28		1.12	2.35
16.50	1.68	2.69	2.99	16.50		
	1.48	1.34	1.34		16.69	16.66
	0.93	0.92	0.92			
	0.61	0.30				
			16.77			
	15.91	16.77				

Appendix II. Estimated size in kilobase pairs (kb) of mtDNA restriction fragments for various restriction enzymes in the shortfin mako (*Isurus oxyrinchus*).

Ava I

(A)	(B)	(C)	(D)	(E)
6.03	6.03	6.03	6.03	6.03
4.99	4.18	4.99	4.99	5.83
2.45	2.45	1.22	2.45	2.45
1.04	1.04	1.22	1.35	1.04
0.85	0.85	1.04	0.70	0.50
0.50	0.70	0.85	-----	-----
-----	0.50	0.50	15.52	15.85
15.86	-----	-----		
	15.75	15.85		

Bcl I

(A)	(B)	(C)
8.23	13.40	8.23
5.11	3.60	5.11
3.60	-----	1.88
-----	17.00	1.20
16.93		-----
		16.42

Bgl I

(A)	(B)	(C)
8.31	8.35	8.02
5.23	7.05	4.36
2.02	-----	2.00
-----	15.40	0.80
15.56		-----
		15.18

BstE II

(A)	(B)
8.07	8.07
4.39	6.96
2.57	2.20
2.20	-----
-----	17.23
17.23	

Dra I

(A)	(B)	(C)
5.53	5.53	5.53
4.41	4.41	4.41
4.17	2.90	4.17
1.64	1.64	2.14
0.50	1.27	-----
-----	0.50	16.25
16.25	-----	
	16.25	

Appendix II. (continued)

Hae II

(A)	(B)
6.84	6.84
6.44	5.71
1.25	1.25
1.25	1.25
	1.12
15.78	
	16.17

Hind III

(A)	(B)
7.08	7.08
6.27	4.28
2.17	2.41
1.20	2.17
	1.20
16.72	
	17.14

Hpa I

(A)	(B)	(C)	(D)
7.25	10.98	7.25	7.25
3.73	3.73	5.58	3.73
3.73	1.85	3.73	3.00
1.85			1.85
	16.56	16.56	0.73
16.56			
			16.56

Stu I

(A)	(B)
6.08	6.08
3.43	2.23
2.23	1.73
1.11	1.70
0.92	1.11
0.92	0.92
	0.92
14.69	
	14.69

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