

**Understanding the Reproductive Behavior and Population Condition of the  
Sandbar Shark (*Carcharhinus plumbeus*) in the Western North Atlantic: A  
Molecular Approach to Conservation and Management**

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Doctor of Philosophy

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by

David Seth Portnoy

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## APPROVAL SHEET

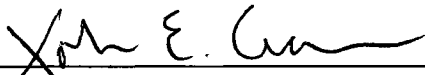
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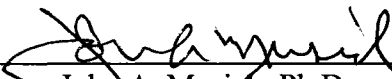
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## **DEDICATION**

I dedicate this work to my loving wife Melissa Megan Boyle Portnoy and my daughter Dacia Flynn Portnoy. They inspire me to achieve great things in everything I do, all the time. I could not have achieved this prestigious accomplishment without them.

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## ABSTRACT

The sandbar shark, *Carcharhinus plumbeus*, has a discontinuous cosmopolitan distribution and is exploited throughout much of its range. In the western North Atlantic, it constitutes the majority of the directed commercial fishery. The stock has declined greatly since the fisheries' inception and has not shown signs of recovery despite the implementation of management practices. Like many highly vagile marine species, it is difficult to obtain information about the sandbar shark through direct observation. Therefore, the goal of this dissertation is to use a molecular approach to examine aspects of behavior and reproduction, providing information useful in conservation and management. To this end, I examine the prevalence of genetic polyandry in the western North Atlantic and estimate effective population size and effective number of breeders for the Delaware Bay and Eastern Shore of Virginia nursery grounds. In addition, I look at patterns of philopatry and reproductive periodicity, while on a worldwide scale, assessing both historical and contemporary gene flow.

Paternity analysis using microsatellite markers reveals that females are likely to mate with multiple males during one reproductive period. Despite the high prevalence of genetic polyandry, no direct benefits are detected. The data, however, do suggest that males benefit by excluding other males from mating, intimating strong intrasexual competition.

The effective number of breeders per nursery ground, estimated using the linkage disequilibrium method, is fairly consistent across years. Comparisons with census size estimates made for Delaware Bay reveal that the two measurements are tightly coupled. The ratio of effective size to census size is 0.45 or higher. This suggests that monitoring of effective population size may be a useful methodology for tracking abundance, and that exploitation may have a direct negative impact on the level of genetic variance.

The results suggest that females may stray between nursery grounds found in Delaware Bay, the Eastern Shore lagoons and Chesapeake Bay, as  $\Phi_{st}$  values are non-significant and kin groups are detected between as well as within samples. However, true kin groups can not be distinguished from erroneous kin groups because sample size is too small and the loci employed do not have enough power. Even so, the results suggest that female reproductive periodicity in this species, thought to be two years, needs to be reevaluated as it appears to be irregular based on these analyses.

Different patterns of historical dispersal and contemporary gene flow are observed when markers with different modes of inheritance are used to evaluate historical phylogeography. The results suggest that, although females show regional philopatry, pulses of female dispersal during the Pleistocene may have created the species' current distribution. This dynamic may have been mediated by the changing distribution of nursery habitat caused by the rise and fall of sea level associated with climate change rather than by fluctuating temperature. This idea is supported by the results, which suggest that male mediated gene flow persists long after female gene flow has stopped.

## AUTHOR'S NOTE

The primary research chapters of this dissertation were written in the format of the journal under which each is in review or to be submitted. These chapters were written in the third person to represent my co-authors. The citations for the chapters are as follows:

### *Chapter 1*

Portnoy, D. S., McDowell, J. R., Thompson, K., Musick, J. A. & Graves, J. E. 2006 Isolation and characterization of five dinucleotide microsatellite loci in the sandbar shark, *Carcharhinus plumbeus*. *Molecular Ecology Notes* **6**, 431-433.

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### *Chapter 5*

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**Understanding the Reproductive Behavior and Population Condition of  
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## INTRODUCTION

## General Biology

The sandbar shark *Carcharhinus plumbeus*, Nardo 1827, was first described based on a specimen caught in the Adriatic Sea. The species is a member of the family Carcharhinidae that contains a minimum of 58 species if the subfamily Sphyrininae is included (Nelson 2006). The genus *Carcharhinus* is the most speciose genus in the family with 31 recognized species (Compagno *et al.* 2005). It has been suggested that the sandbar shark is part of a monophyletic group of large carcharhinids, including *C. altimus*, *C. falciformis*, *C. longimanus*, *C. perezi*, *C. obscurus*, and *C. galapagensis* that feature an interdorsal ridge (Naylor 1992). This group may also include *Prionace glauca*, the blue shark. The ridge-backed clade is thought to have a fairly recent origin, and the fossil record supports this notion as some modern carcharhinids appear as early as the Lower Eocene, while *Prionace* does not occur until much later in the Pliocene (Capetta 1987). All the members of this group, except *C. perezi*, have cosmopolitan distributions and can often be found parapatrically, partitioned in the environment by parameters like depth and temperature (Musick *et al.* 2004).

Like other members of the ridge-backed group, the sandbar shark, has a discontinuous cosmopolitan distribution. It is found coastally within warm-temperate to tropical waters. While the species has a circumglobal distribution, it is absent from the expanse of Oceania between New Caledonia and Hawaii (Compagno *et al.* 2005) and is likely absent in the eastern Pacific (J. Musick, personal communication). The species is both long-lived and slow to reach maturity, reaching lengths as great as 250cm TL. Longevity has been estimated to be at least 30 years with time to maturity varying from 15-16 years in the western North Atlantic (Sminkey and Musick 1995) to 8-10 years in

Hawaiian waters (Romine *et al.* 2006). It is placental viviparous and has small litter sizes (4-16) with fairly large (~60cm TL), well developed pups (Sminkey and Musick 1996).

In the western North Atlantic, mating occurs in the late spring and early summer. At this time aggregations of male and female sharks are encountered over the outer shelf off Florida. Although mating has not been observed directly, females with fresh mating wounds and seminal fluids in their reproductive tracts are often caught in the area (Springer 1960, Pratt 1993). Springer (1960) observed that only ~20% of these females were carrying yolked ova or embryos, with the rest in an apparent quiescent phase, and came to the conclusion that females exhibit a two year reproductive cycle, a conclusion also reached by Joung and Chen (1995). Like many other shark species, the sexes are segregated at all other times of the year (Springer 1967). Males remain offshore, while pregnant females migrate from the mating grounds to coastal nurseries. The Chesapeake Bay, the Virginian Eastern Shore lagoons and Delaware Bay have been identified as principal nursery grounds (Grubbs and Musick 2007) with embayments from North Carolina to Florida, north of Cape Canaveral, serving as smaller, secondary nursery grounds (Snelson and Williams 1981, Castro 1993). Smaller nurseries have also been found as far north as Cape Cod, MA and to the west in the Gulf of Mexico (Castro 1993, Carlson 1998).

For sharks with limited lifetime reproductive opportunities and small litter sizes, well protected nurseries are important for increasing juvenile survival (Branstetter 1990). For sandbar sharks, the benefit of increased juvenile survival must be relatively large because it balances the additional parental cost to females associated with internal gestation and migratory behavior (Simpfendorfer and Milward 1993). Coastal nurseries

are important for growing juveniles as they are rich in prey species (Medved *et al.* 1985), but more importantly, they decrease juvenile mortality by providing pups with a safe haven from large elasmobranch predators like the bull shark, *Carcharhinus leucas* (Springer 1960), as well as adult conspecifics. Juvenile sandbar sharks are tied to this habitat, migrating from the outer shelf off North Carolina to their natal nursery areas every summer for the first 4-12 year of life (Grubbs *et al.* 2007, McCandless *et al.* 2007).

### The Fishery

The sandbar shark is the target of commercial fisheries throughout most of its range (McAuley *et al.* 2007) not only because it is valued for its palatable meat, but also for its large fins. The species comprises almost 2/3 of the United States commercial shark catch in the western North Atlantic (Grubbs 2001). It is taken in a directed longline fishery and is also captured incidentally by other fisheries (NOAA 2001). Musick *et al.* (1993) noted declining abundance of the species between 1974 and 1991 and called for more stringent management. That same year the species began to be managed as part of the large coastal complex in the Atlantic Shark Fisheries Management Plan (NMFS 1993) and by 1999 became part of the Highly Migratory Species Fishery Management Plan (NMFS 1999). In the face of fisheries driven by the high demand for fins in Asian markets, the Shark Finning Prohibition Act was signed into law in 2000 (NMFS 2004).

Declines in shark stocks in response to fishing are not unprecedented, as soupfin shark (*Galeorhinus galeus*), and spiny dogfish (*Squalus acanthias*) stocks in the 1940s and 1950s crashed within a period of decades under the weight of heavy fishing pressure (Ripley 1946, Olsen 1959, Aeson 1964, Anderson 1990). Likewise, stocks of porbeagle

(*Lamna nasus*), sandtiger (*Carcharias taurus*), and dusky shark (*Carcharhinus obscurus*) have more recently been severely depleted in the western North Atlantic (Musick *et al.* 2000). Given the reproductive mode, longevity, and slow approach to maturity in *C. plumbeus*, it is not surprising that models indicate that the stock can only be fished at very low levels to prevent decline (Sminkey and Musick 1996, Brewster-Geisz and Miller 2000), and that the species has an extremely low rebound potential (Smith *et al.* 1998). Aware of these concerns, NOAA amended the Highly Migratory Species Fishery Management Plan in 2003, taking the sandbar shark's life history into account. While there was optimism about the stock's recovery, attempts to characterize the stock's population size and trajectory based on fishery dependent data sets gave somewhat contradictory results (Cortes *et al.* 2002). The latest assessment indicates that the stock is still not recovering (SEDAR 2006) and NOAA (2007) has suggested that fishing be limited to only those commercial vessels involved in research. This suggestion has met with much resistance by fishermen whose livelihoods depend on this fishery.

#### A Molecular Approach to Conservation and Management

Despite being one of the more thoroughly studied elasmobranchs, many questions still remain that are vital to conservation and management of the sandbar shark, and molecular techniques offer a unique prospective on these issues. The power of such an approach is that it allows one to investigate aspects of behavior, demography and population structure that may be inaccessible by observational research (Awise 1998). In addition, the same suite of markers can be applied to questions ranging from individual behavior all the way up to historical biogeography.

This dissertation has been divided into five chapters that use data generated through multi-locus microsatellite genotyping and mtDNA control region sequencing to gain an understanding of several aspects of sandbar shark reproduction and behavior that may be important for conservation and management. The first chapter deals with the technical nature of designing and evaluating species-specific microsatellite markers and appeared in the journal *Molecular Ecology Notes* in 2006. The second chapter takes a fine scale look at individual male contribution to litters, using microsatellite markers to better understand patterns of genetic polyandry and mating systems in sandbar sharks. It appeared in the journal *Molecular Ecology* in 2007. The third chapter uses microsatellite data to evaluate the effective population size of two of the more important nursery grounds in the mid-Atlantic and to elucidate the number of breeders using these areas while examining possible genetic consequences that fisheries may impose on fished elasmobranch stocks. It has been submitted to *Proceedings of the Royal Academy of Sciences London B*. The fourth chapter uses microsatellite and mtDNA data to examine the fidelity of philopatry to nursery grounds and to reassess female reproductive periodicity as both have a direct impact on management decisions. The fifth and final chapter uses microsatellite and mtDNA data to describe the global phylogeography of the species, to not only elucidate important patterns of historical biogeography but to understand contemporary gene flow.

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## Chapter 1

# Isolation and Characterization of Five Dinucleotide Microsatellite Loci in the Sandbar Shark, *Carcharhinus plumbeus*.

**Abstract:**

Five dinucleotide markers were isolated and optimized from a microsatellite-enriched genomic library obtained from the sandbar shark, *Carcharhinus plumbeus*. Genotypic distributions of all markers were found to be in conformance with the expectations of Hardy-Weinberg equilibrium with 4 to 39 alleles present per locus. We amplified these loci in two female sharks and their litters. A maternal allele was recovered at each locus in all progeny indicating reliable amplification. More than two paternal alleles were recovered across both litters indicating genetic polyandry. Additionally, these markers were amplified across ten carcharhiniform species to examine their utility in other studies.

Sandbar sharks are large coastal carcharhinids with a cosmopolitan distribution. They have a 30 year lifespan and take 15 years to reach maturity (Sminkey & Musick 1995). In the western North Atlantic, sandbar sharks are a major component of the commercial shark fishery and are caught recreationally (NMFS 2001). Due to the species' slow growth and late maturity, along with the pattern of exploitation, it is listed as "conservation dependent" (IUCN 2004). Previous studies based on mitochondrial DNA and microsatellites suggest a single western North Atlantic stock (Heist *et al.* 1995, Heist & Gold 1999). Information essential for conservation and management, such as the level of female philopatry to nursery grounds and the magnitude of gene flow between disjunct populations, has not been acquired because markers lacked sufficient variability. Here we characterize five highly polymorphic dinucleotide microsatellite loci.

Sandbar shark muscle was powdered by grinding in liquid nitrogen and high molecular weight DNA was extracted following the protocols of Sambrook and Russell (2001). Microsatellites were isolated following the protocols of Hamilton *et al.* (1999) with minor modifications. Briefly, genomic DNA was digested using *RsaI*, *BstUI* and *XmnI* (New England Biolabs) simultaneously, dephosphorylated, and resulting fragments were ligated to SNX (Invitrogen) linkers in the presence of *XmnI*. Biotinylated (GT)<sub>12</sub> (Invitrogen) was used to perform subtractive hybridization reactions at 75°C overnight following Kijas *et al.* (1994). After hybridization, Streptavidin MagneSphere<sup>®</sup> Paramagnetic Particles (Promega) were added at a concentration of 1 mg/ml and the solution was agitated for several hours in a shaker bath at 43°C. Beads were washed twice with 200 µl of 2X SSC, 0.1% SDS and four times with 200 µl of 1X SSC, 0.1%

SDS for five minutes per wash. Beads were separated from the solution between washes using a MagneSphere<sup>®</sup> Magnetic Separation Stand (Promega). Microsatellite enriched DNA was eluted by adding 60  $\mu$ l of preheated T.E (10mMTris, 0.1mM EDTA), incubating at 95°C for 10 minutes and separating the solute from the beads. The recovered single stranded DNA was amplified using a forward SNX linker as a primer. The resulting double-stranded products were ligated into PCR 2.1<sup>®</sup> vector (Invitrogen) and transformed into Top10 One Shot<sup>®</sup> (Invitrogen) competent *E. coli* cells. Colonies containing inserts were selected following manufacturer protocols and suspended in 100  $\mu$ l of sterile water. Suspensions were boiled for five minutes and centrifuged for two minutes at 16,000 g to extract plasmids.

Ten  $\mu$ l PCR reactions using M13F and M13R primers were used to screen the library for microsatellite inserts. All PCR reactions were run on a PJC-200 thermocycler (MJ research). Reaction conditions consisted of a denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Recombinant plasmids containing inserts of at least 100 bp were re-amplified at a volume of 50  $\mu$ l as above and used as template for sequencing reactions with the Thermosequence Primer Cycle Sequencing<sup>™</sup> Kit (Amersham). Reactions were electrophoresed on a 3.7% polyacrylamide gel using a LiCor global IR<sup>2</sup> system with either IRD-700 labeled M13R or IRD- 800 labeled M13F primers (LiCor). Locus-specific primers were designed using the "find PCR primer pairs" option in the analysis menu of Mac Vector 8.0 (Accelrys).

189 inserts were sequenced, 35 contained repeats and 27 primer pairs were ordered. Five primer pairs (Cpl-53, Cpl-90, Cpl-128, Cpl-166, and Cpl-169) reliably amplified a single locus; no more than two bands were present on polyacrylamide gels with labeled primers. Products resulting from these five primer pairs were subsequently cloned and re-sequenced for validation purposes. These five loci were tested on 47-55 sandbar sharks. Five  $\mu\text{l}$  reactions contained 20 mM Tris-HCL (pH 8.4), 1.2-1.5 mM  $\text{MgCl}_2$ , 0.001mg/ $\mu\text{l}$  BSA, 0.2mM dNTP mix, 20 pmol of primer (except Cpl 128, which contained 10 pmol of primer), 0.2  $\mu\text{l}$  of template and 0.025 units/ $\mu\text{l}$  of *Taq* polymerase (Invitrogen). Forward primers were labeled with IRD-800 or IRD-700 fluorescent dye (LiCor). Reaction conditions consisted of a denaturation of 95°C for 4 min followed by 25-40 cycles of 94°C for 1 min, the appropriate annealing temp (Table 1) for 0.5-1min, 72°C for 1 min, followed by 72°C for 10 minutes. The locus Cpl-53 was amplified with a touchdown protocol of 95°C for 1 min followed by 3 iterations of 5 cycles at 94°C for 1min, annealing (62°C, 61°C, 60°C) for 1 min, 1 min at 72°C, followed by 25 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, followed by an extension at 72°C for 10 minutes. Products were separated on 25cm 6.5 % polyacrylamide gels using a LiCor 4200 Global IR<sup>2</sup> system. A 50-350bp size standard was run in the first, middle, and last lanes of each gel and with locus-specific standards in every 8th lane. Alleles were scored using Gene ImagIR 4.05 (Scanalytics). GENEPOP 3.4 (Raymond & Rousset 1995) was used to analyze conformance to Hardy-Weinberg equilibrium and test for linkage disequilibrium. All loci were cross-amplified in ten other species of Carcharhiniform sharks using a gradient thermocycler with annealing temperatures between 52°C and

65°C. Products were electrophoresed on 2% agarose gels to assess amplification success (Table 2).

All loci were unlinked and polymorphic, with between 4 and 39 alleles present, conformed to the expectations of Hardy-Weinberg equilibrium (Table 1). Microsatellite loci were used to genotype two female sandbar sharks and their respective litters. Maternal alleles were recovered in every pup in both litters, indicating reliable amplification for all primer pairs. More than two paternal alleles were noted within each litter at all loci with the exception of Cpl-53 in one of the litters. This demonstrates genetic polyandry in *C. plumbeus*. Further analyses are necessary to determine the prevalence of this reproductive behavior. Using the sandbar-specific primer pairs, all loci could be amplified in at least one other Carcharhiniform species (Table 2). Since products were separated on agarose gels and inspected by eye, accurate determination of allele size or number was not possible.

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Table 1: Five microsatellite loci developed for sandbar sharks includes: locus name, GenBank accession number, primer sequence, repeat motif, annealing temperature (Ta), dye label (DL), observed size range, number of alleles observed, observed heterozygosity ( $H_o$ ) vs. expected heterozygosity ( $H_e$ ), conformance to Hardy-Weinberg equilibrium (Phw), number of individual genotyped (#)

Locus	GenBank No.	Primer Sequence 5'-3'	Motiff	Ta (C°)	DL	Size Range (bp)	alleles	Ho(He)	Phw	#
Cpl 53	DQ191806	F CAAGCAGGCAGCTAAGAG R CATTTCGTCTGTATAGAGCATAAG	(TG)18.	(62-60) 57	IR-800	166-186	4	.63(.57)	0.862	50
Cpl 90	DQ191807	F GTTGTTGCCTTGTCTTCAATCG R TGTGCACTGTGTCTCTGTGTGCC	(AC)24	56	IR-700	214-278	26	.88(.93)	0.109	51
Cpl 128	DQ191805	F GCTGTGATCTTTGCTGATTGAGC R GGATGGTGGATTGTGGATTTG	(CA)13TA(CA)13	65	IR-800	216-254	15	.90(.87)	0.636	50
Cpl 166	DQ191809	F TGGACATGACAATTACAGCACAGG R CTGTTTACAACCTCCCTGGAGTGC	(GT)17	63	IR-800	223-325	39	1(.96)	0.958	47
Cpl 169	DQ191810	F TGACACAACCATTTATTCCCACG R GGTTTCCTTGAGTGAAAGAGAGAGC	(TG)42	64	IR-700	107-209	29	.92(.93)	0.650	55

Table 2: Results of cross amplification for other Carcharhiniform sharks: *Carcharhinus longimanus* (C .lon), *Carcharhinus limbatus* (C. lim), *Carcharhinus brevipinna* (C. bre.), *Carcharhinus falciformis* (C. fal), *Carcharhinus obscurus* (C. obs), *Galeocerdo cuvier* (G. cuv), *Rhizoprionodon terraenovae* (R. ter), *Prionace glauca* (P. gla), *Mustelus canis* (M. can), *Sphyrna lewini* (S. lew). Numbers next to species designation indicate number of individuals.

Marker	C. lon(7)	C. lim(3)	C. bre(3)	C. fal(4)	C. obs(17)	G. cuv(2)	R. ter(1)	P. gla(10)	M. can(2)	S. lew(3)
cpl-53	*(52-56)	/	/	/	/	0	0	*(52-55)	*(55-59)	*(56-58)
cpl-90	*(56-62)	*(60-63)	*(59-63)	*(59-63)	*(60-63)	/	0	*(56-60)	/	0
cpl-128	*(52-56)	0	0	*(52-56)	*(52-56)	0	/	*(56-60)	0	*(52-56)
cpl-166	*(56-62)	0	*(52-55)	0	/	0	0	0	0	/
cpl-169	*(54-56)	/	/	*(59-62)	*(54-56)	0	0	*(53-56)	0	0

\*(#) indicates temperature range over which appropriately sized amplicons appeared, / indicates some nonspecific amplification requiring further optimization, 0, indicates smear or no product.

## Chapter 2

Genetic Polyandry and Sexual Conflict in the Sandbar Shark, *Carcharhinus plumbeus*, in the Western North Atlantic and Gulf of Mexico

**Abstract:**

To investigate patterns of polyandry in the sandbar shark (*Carcharhinus plumbeus*), 20 pregnant females were sampled from the western North Atlantic and Gulf of Mexico. Five species-specific microsatellite markers were used to genotype each shark and its litter. Of 20 litters, 17 (85%) were shown to have multiple sires. In multiply sired litters, the estimated minimum number of sires ranged from 2 to 5 with an average of 2.3 males per litter. Regression analysis did not demonstrate a significant relationship between female reproductive success and female body size or sire number and female body size. There was a high incidence of reproductive skew noted in litters, and two groups of males with significantly different mean reproductive success were observed. Analyses using Bateman's principles suggest that there is less direct benefit for females that acquire multiple mates than for males who bias paternity within litters. In light of past morphological and behavioural studies, these data suggest that patterns of polyandry in elasmobranchs may be determined by coercive mating, and that breeding behaviour has likely evolved in the context of sexual conflict.

## Introduction:

Studies using high resolution molecular markers have revealed that genetic polyandry is common across taxa (see Birkhead and Møller 1998 for a review). In aggregate spawning species with external fertilization, such as many bony fishes and amphibians, the presence of multiple sires per clutch is expected (Myers & Zamudio 2004; DeWoody & Avise 2001). However, polyandry has been demonstrated to be common in taxa with internal fertilization (e.g. mammals and birds) which were previously considered to be monogamous or polygynous (Gibbs *et al.* 1990; Carling *et al.* 2003; Goetz *et al.* 2003; Yamaguchi *et al.* 2004). These findings have lead many researchers to examine the potential benefit polyandry may provide to females that actively accept multiple copulations despite the associated costs.

Females may benefit directly or indirectly from multiple matings. Direct benefits, which increase reproductive success, may take the form of nutritive gifts that can be invested in the production of ova, as in insects (e.g. the decorated cricket, Sakaluk *et al.* 2006) or, increased sperm volume in species such as the American lobster (Gosselin *et al.* 2005). Species that are less sperm or energy limited, like the redwinged black bird or the freshwater sunfish, may benefit directly from polyandrous mating through shared parental care or territory usage (Gray 1997; Avise *et al.* 2002). Indirect genetic benefits do not affect reproductive success but may increase survivorship or reproductive success of offspring (Zeh & Zeh 2001). These benefits include increased additive genetic variance in progeny, bet-hedging in unstable environments, pre-copulatory or post-copulatory trading-up, and post copulatory defence against genetic incompatibility (Zeh

& Zeh 1997; Newcomer *et al.* 1999; Jennions & Petrie 2000; Tregenza & Wedell 2000; Simmons 2003). However, many studies have been unable to demonstrate female benefit from polyandrous mating (Byrne & Roberts 2000; Garner & Schmidt 2002) raising doubt that genetic benefits alone can promote polyandry (Yasui 1998).

Mating partners that are genetically distinct have different ideal reproductive outcomes (Lessells 1999) which can lead to sexual conflict and greatly influence mating behaviour (Zeh & Zeh 2003; Parker 2006; Tregenza *et al.* 2006). A female's optimal mating frequency is determined by the balance between the costs associated with mating and the benefits of polyandry. Males, who generally produce greater amounts of energetically less costly gametes, can optimize their fitness by increasing the number of matings in which they participate (Bateman 1948; Arnqvist & Nilsson 2000) and/or by biasing sperm usage in multiply mated females. In situations where there is conflict over mating frequency, males may attempt to coerce resistant females into additional matings (Partridge & Hurst 1998). This dynamic may lead to antagonistic coevolution, and in species where males have gained the advantage, the number of matings may be maintained above the female optima (Rowe & Arnqvist 2002). These superfluous matings increase the rate of genetic polyandry, often at a cost to female fitness (Warner *et al.* 1995; Byrne & Roberts 1999; Maklov and Lubin 2004).

It is preferable to investigate changes in fitness associated with polyandry using controlled laboratory experiments, in which the number of matings can be carefully manipulated while benefits and costs to both sexes can be measured (Jones & Avise 2001). However, large vertebrates such as elasmobranchs (sharks, skates, and rays), do not lend themselves to such manipulation due to the difficulty of maintaining captive

populations and aberrant behaviour resulting from the stress of captivity (Henningsen *et al.* 2004). Alternatively, high resolution microsatellite markers allow for kinship analyses (Blouin *et al.* 1996; Fiumera *et al.* 2001; Jones and Arden 2003). In situations where entire litters can be genotyped, detailed information about male and female reproductive output can be collected. Comparative approaches utilizing phylogenetic information can then be used to investigate the adaptive significance of reproductive behaviour (Harvey & Pagel 1991).

Elasmobranchs are a basal vertebrate lineage with internal fertilization. Some elasmobranchs feature prolonged maternal care in the form of long gestation periods and reproductive cycles greater than one year (Carrier *et al.* 2004). Mating is physically costly to females, as copulation requires males to grasp and hold on to females with their jaws (Pratt and Carrier 2001). Despite the substantial cost, varying levels of polyandry have been observed in most species examined. In the nurse shark, *Ginglymostoma cirratum* (Ohta *et al.* 2000; Saville *et al.* 2002), and the lemon shark, *Negaprion brevirostris* (Feldheim *et al.* 2002; Feldheim *et al.* 2004), the majority of litters examined had multiple sires. In contrast, the majority of litters in the bonnethead, *Sphyrna tiburo*, had one sire (Chapman *et al.* 2004) as did the single litter examined in the banded houndshark, *Triakis scyllium* (Ohta *et al.* 2000). The balance of costs and benefits that have led to differences in the rate of polyandry across these species is not entirely clear. A comparison of rates of polyandry, demography and life history across related shark species will help elucidate the evolutionary implications of polyandry in elasmobranchs and may be instructive for further inquiries into the fitness consequences of polyandry in species where mating incurs significant cost.

The sandbar shark, *Carcharhinus plumbeus*, is part of a monophyletic unit (Carcharhinidae), with *Negaprion brevirostris* and *Sphyrna tiburo* (Naylor 1992). Therefore patterns of polyandry in sandbar sharks are of interest from a comparative evolutionary perspective. This species is also of interest from a conservation perspective because it is cosmopolitan and exploited throughout much of its range (Compagno 1984). The western North Atlantic population, which extends into the Gulf of Mexico (Bigelow & Schroeder 1948; Springer 1960; Heist *et al.* 1995), is a primary target of the commercial shark fishery (Burgess & Morgan 2002). Like other carcharhinids, it is long lived, slow to mature, and has a low fecundity, making its lifetime reproductive output more similar to that of a cetacean than a bony fish (Smith *et al.* 1998). Understanding factors that affect levels of polyandry may be important in maintaining viable populations in the face of exploitation (Martinez *et al.* 2000; Rowe & Hutchings 2003).

We characterized the prevalence of multiple paternity in sandbar sharks in the western North Atlantic using highly polymorphic microsatellite markers. We investigated whether there is direct female benefit to genetic polyandry by examining the relationship between mating success (the number of sires) and female reproductive success (number of offspring) (Bateman 1948; Jones *et al.* 2000; Jones *et al.* 2002). As an alternative, we examined whether female reproductive success simply varied with size. Since sandbar shark mating is violent in nature (Springer 1960), we hypothesized that small and large females might exhibit different mating rates, which would be reflected in sire number. If large females can better absorb the costs of mating and benefit indirectly from genetic polyandry, then the number of sires would be positively correlated with female size. Alternatively, if indirect benefits are small, and large females can resist coercive mating

better than smaller females, the number of sires would be negatively correlated with female size. Since polyandry creates a forum for sperm competition, even when male success comes at the expense of female fitness (Chapman *et al.* 1995), we investigated male fitness by examining the relationship between male reproductive success and the number of competing sires.

#### Materials and Methods:

##### Collection and Genotyping

Twenty pregnant sandbar sharks were collected in the western North Atlantic Ocean and Gulf of Mexico through two fishery independent longline surveys and the Florida Museum of Natural History's Commercial Shark Fishery Observer Program. These animals were considered to be sampled from a single population based on the results of prior molecular analyses and tagging studies (Heist *et al.* 1995; Musick unpublished data). Fork length (FL), measured from the tip of the snout to the fork of the tail, was determined for each shark. The paired uteri were dissected from each female, placed on ice, and frozen upon return to the laboratory for later analysis. All pups were removed from the uteri and measurements of pup FL were taken.

Tissue samples, in the form of fin clips, were taken from all pups. Either fin clips or uterine tissue were taken from adult female sharks for genetic analysis. Tissue was stored in DMSO buffer (Seutin *et al.* 1991) or 95% ethanol at 4°C. DNA was subsequently extracted using the Chelex protocol described by Estoup *et al.* (1996). After 2 minutes of centrifugation at 16,000g, 0.3µl of the supernatant was used directly as a

template for 5 µl PCR reactions. Five highly polymorphic microsatellite markers (Cpl-90, Cpl-128, Cpl-132, Cpl-166, Cpl-169) isolated from an enriched genomic library were amplified using IR-700 and IRD-800 labelled forward primers for each mother and her litter (Table 1). Descriptions of the primers and PCR conditions are reported elsewhere for four of the markers (Portnoy *et al.* 2006). The fifth marker Cpl-132 (F: CTC CCT TCC CTA CCA TAT TTC C, R: AAT ACA GGA GGC TTT GCA CGC, Genbank accession #: DQ191808) was optimized for this study. Cpl-132 reactions contained 20 mM Tris-HCL (pH 8.4), 1.2 mM MgCl<sub>2</sub>, 0.001mg/µl BSA, 0.2mM dNTP mix, 20 pmol of primer. This marker required a step-up PCR protocol. The reaction conditions consisted of a denaturation at 95°C for 4 min followed by 5 cycles at 94°C for 1 min, 58°C for 0.5 min and 72°C for 1 min, followed by 25 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. All amplicons were electrophoresed through 25 cm 6.5 % polyacrylamide gels using a LiCor 4200 Global IR<sup>2</sup> system. A 50-350 bp size standard was run in the first, middle, and last lanes of each gel and locus-specific standards were run in every 8th lane. Alleles were scored manually with the aid of Gene ImagIR 4.05 (Scanalytics). Twenty percent of samples were randomly selected and rescored to ensure accurate scoring.

#### Genetic Data Analyses

Allele frequencies were calculated for each locus with FSTAT (Goudet 2001) using 67-73 adult individuals, including the 20 adults collected for this study, from throughout the species range in the western North Atlantic and Gulf of Mexico. Conformance to the expectations of Hardy-Weinberg equilibrium was calculated for each

locus in GENEPOP (Raymond & Rousset 1995) using exact tests with 10,000 iterations. These same individuals were used to calculate the probability of excluding incorrect sires, given a known maternal genotype, for each individual locus and across all loci in Gerud 2.0 (Jones 2005) using the methodology of Dodds *et al.* (1996). The probability of detecting multiple paternity (PrDM) was calculated using PrDM software (Neff & Pitcher 2002), which only allows the user to input frequency data for 30 alleles per locus. Since Cpl-166 and Cpl-169 both have more than 30 alleles, low frequency alleles that did not appear in the maternal genotype were binned two at a time until only 30 states were left. For each litter, scenarios specific to the maternal genotype with different levels of paternal skew were considered. For example, for a monogamous litter with 10 offspring we evaluated the PrDM under several scenarios in which the litter actually had two sires. In each scenario we used a different ratio of paternal contribution

Genotypic arrays were visually inspected to ensure that all progeny shared at least one allele at each locus with their mother. The number of paternal alleles for each locus across a litter was then summed. A litter was considered polyandrous if two or more loci across a litter had three or more paternal alleles. Allele counts for each locus allowed for an initial estimate of the number of sires. For litters in which all loci had only two paternal alleles, Fisher's exact tests were used to determine whether loci conformed to the expectations of Mendelian segregation in a monogamous mating. Gerud 2.0 (Jones 2005) was then used to estimate the minimum number of fathers that sired a litter and the number of progeny per sire from the array of genotypes expressed by the female and her progeny. For cases in which no unique solution was found, up to fifty solutions with the highest priority scores were ranked. Colony 12 (Wang 2004), a program that clusters full

sibling families within half sibling families using multi-locus gene arrays, was also used to estimate the number of fathers that sired a litter and their relative contribution. Paternal genotypes reconstructed by both programs were examined to determine whether any sires had contributed to multiple litters. Reconstructed fathers were screened for the presence of multiple alleles across loci that were in high frequency in the population, as this may indicate multiple males being treated as one (Myers and Zamudio 2004). Estimates of sire number and patterns of paternal contribution obtained by the different algorithms were subsequently compared to ensure more robust results.

Possible relationships between female reproductive success (litter size) and body size (FL) as well as the number of sires and female size were determined through linear regression analysis. Chi-square analysis was used to test the null hypothesis that male reproductive success was random and would therefore conform to a Poisson distribution (Zar 1999). For each litter, the male with the greatest reproductive success in terms of number of offspring sired was designated as the most successful male. Bateman (1948) stated that variance in reproductive success was indicative of intrasexual selection and that the correlation between reproductive success and mate number was the cause of this selection. Therefore, by using Bateman's principles, the direct benefit for females who mate multiply can be compared to the benefit for males that limit additional male contribution to litters. It is important to note that although the latter relationship was not expressly discussed by Bateman, his principles can be applied because the correlation between reproductive success and number of additional sires still measures the fitness component of selection, and the variance in reproductive success still measures its strength. To make these comparisons, reproductive success was regressed against

mate/sire number for females and the most successful males. Point estimates and confidence intervals of the slopes (B) were then used to examine relative benefit (Arnold 1994; Arnold & Duvall 1994; Jones *et al.* 2002). The standardized variances in reproductive success (I) were calculated by dividing the variance in reproductive success by the squared mean of reproductive success for each sex, allowing for the comparison of the strength of selection on each sex (Wade 1979; Wade & Arnold 1980; Jones *et al.* 2002).

#### Results:

The distribution of genotypes at all loci conformed to the expectations of Hardy-Weinberg equilibrium (Table 1). The number of alleles present at each locus ranged between 12 and 45 (Table 1). Exclusion probabilities were high for each locus and the cumulative exclusion probability was greater than 0.99 (Table 1). A low frequency null allele (0.014) was discovered in two mothers and their litters at locus Cpl-169 (litters A and C). All pups in both of these litters amplified at least one allele at this locus. In addition, within litter allele counts were consistent between Cpl 169 and the other four loci. Since Cpl-169 also conformed to the expectations of Hardy-Weinberg equilibrium the use of this locus did not bias our estimation of paternal contribution.

Genetic polyandry was detected in seventeen of twenty litters (85%) by allele counts. Litters A, D and R had four or fewer parental alleles for each locus examined, consistent with genetic monogamy. Fisher's exact tests were non-significant in these litters indicating that all loci conformed to expectations of Mendelian segregation,

supporting the conclusion that they were genetically monogamous. PrDm was lowest in the genetically monogamous litters (65%) when reproductive skew was assumed to be high (12-1), but increased rapidly as skew was decreased. Of the polyandrous litters, the number of sires per litter estimated by Gerud 2.0 varied between two and four, while Colony estimated between two and five sires per litter. The average numbers of sires per litter as estimated by Gerud 2.0 and Colony were 2.30 and 2.65, respectively. Gerud 2.0 produced a unique paternity solution in seven litters. For the remaining ten litters, priority scores produced by Gerud 2.0 were used to rank scenarios. Only litter J and K had more than fifty solutions prior to ranking. For seven of these litters, all solutions predicted the same number of progeny per sire but differed in paternal genotypes. Litters J and Q had two solutions with different progeny per sire ratios. However, the same progeny per sire ratios appeared in the majority of solutions, most of which had higher ranking priority scores. Only litter O resulted in more than two solutions with differing progeny per sire ratios. Even so four of six solutions for this litter predicted the most successful male sired six of the pups (Table 2). Colony results were the same as the highest ranking Gerud results for eight of the polyandrous litters. In eight of the remaining nine litters, Colony predicted the same number of progeny for the most successful sire but more total sires or different paternal contribution ratios. For litter J, Colony predicted fewer offspring for the most successful sire than Gerud (Table 2). No reconstructed male genotypes appeared more than once across litters and none had an overabundance of high frequency alleles.

Gerud 2.0 and Colony results showed similar trends and significance in most subsequent analyses, therefore, only the results using Gerud 2.0 data are presented below. The regression of reproductive success as a function of maternal fork length, had a slope

that was not distinguishable from zero with fairly tight 95% confidence intervals ( $B = -0.015$ ,  $P = 0.80$ ,  $CI\ 95\% = -0.14 < B < 0.11$ ). The slope of relationship between the number of sires and female length was also not significantly different than zero ( $B = -0.027$ ,  $P = 0.26$ ,  $CI\ 95\% = -0.076 < B < 0.022$ ). The distribution of reproductive success across all 46 males did not conform to the expectations of a Poisson distribution ( $df = 7$ ,  $\chi^2$  value = 25.38,  $P < 0.01$ , Fig. 1). When the data were partitioned into the reproductive success of the most successful males in each litter versus other sires, the success of the most successful males conformed to the expectations of a Poisson distribution ( $df = 7$ ,  $\chi^2$  value = 4.42,  $P > 0.75$ , Fig. 1). The mean reproductive success of the most successful males was 6.3 pups per litter while the mean success for all other males was 2.4 pups per litter (t-test,  $df = 22$ ,  $P < 0.001$ ).

Slope estimates for the regression of female reproductive success as a function of sire number differed depending on whether Gerud or Colony results were used (Gerud:  $B = 0.98$ ,  $95\% CI = -0.11 < B < 2.1$ ; Colony:  $B = 0.43$ ,  $95\% CI = -0.46 < B < 1.31$ , Fig. 2), however, neither slope was significantly different than zero (Gerud:  $P = 0.076$ , Colony:  $P = 0.32$ ). The regression of the most successful males reproductive output against the number of sires per litter showed an inverse relationship, with consistent estimations of slope between Gerud and Colony (Gerud:  $B = -1.30$ ,  $95\% CI = -2.42 < B < -0.19$ ; Colony:  $B = -1.12$ ,  $95\% CI = -1.95 < B < -0.30$ , Fig. 2). In both cases, the slopes were significantly different than zero (Gerud  $P = 0.024$ ; Colony  $P = 0.01$ ). The standardized variance in reproductive success was higher for the most successful males (Gerud:  $I = 0.13$ ; Colony:  $I = 0.14$ ) than females ( $I = 0.05$ ).

## Discussion:

Genetic polyandry occurs with high frequency in *C. plumbeus*. Of the 20 litters examined, 17 (85%) had multiple sires. This level of polyandry is consistent with some previous studies which reported 86% polyandry in *N. brevirostris* and 100% polyandry in *G. cirratum* (Ohta *et al.* 2000; Saville *et al.* 2002; Feldheim *et al.* 2004). In *S. tiburo* however, genetic polyandry was found in less than 19% of the litters examined (Chapman *et al.* 2004). Average litter sizes of polyandrous females were approximately 15 (N = 2) in *N. brevirostris* (Feldheim *et al.* 2002) and 29 (N = 3) in *G. cirratum* (Ohta *et al.* 2000; Saville *et al.* 2002). For *S. tiburo*, multiply sired females had an average litter size of 14 (N=4) with significantly larger litters than monogamous mating females (Chapman *et al.* 2004). Despite smaller average litter size in *C. plumbeus* (just over 9, N = 20), polyandry was the dominant reproductive mode. Even the smallest litter (4) had multiple sires. In addition, male reproductive success was highly skewed within litters. Of the 17 polyandrous litters examined, nine had one male siring at least 60% of the total progeny. A similar pattern was observed in *S. tiburo* (Chapman *et al.* 2004) where high skew in male success in polyandrous litters was also present.

While the present study was unable to distinguish whether the mating system in *C. plumbeus* is truly polyandrous or is in fact polygynandrous, previous observational and experimental approaches in other shark species have revealed polygyny (Feldheim 2004; Pratt and Carrier 2001). Theoretically, polygyny increases the fitness of any male able to

sire multiple litters (Bateman 1948); therefore we feel it is likely that polygynous mating occurs in the sandbar shark.

We were unable to detect a relationship between female size and reproductive success. This may be due, in part, to sample size and/or the small range in litter sizes (between 4 and 13) observed in this study. Our point estimate of the slope, however, was very small and negative (-0.015 pups/cm). Taken literally this slope would mean that a female shark that grew 70 cm would have a decrease in reproductive success of one pup. Given that the species matures at 150 cm fork length and the largest females are around 215 cm in fork length (Casey & Natanson 1992; Sminkey & Musick 1995) this point estimate lacks biological meaning. Similarly, we were unable to find a relationship between female size and sire number. Once again, the slope was quite small and negative (-0.027 sires/cm) lacking biological meaning throughout most of the 95% confidence interval. These data suggest that size is unrelated to the number of sires either because female sandbar sharks show no preference for number of matings or are unable to control mating frequency.

The development and use of highly variable microsatellite markers is critical to this type of study because the increased genetic resolution offsets the decreased probability of detecting sires when the number of offspring sampled are small (Neff & Pitcher 2002). In addition, the molecular markers provided fairly consistent results when estimating paternal contribution using programs that use different algorithms to estimate paternal contribution. The major difference in the output between the two programs is due to how each algorithm treats two unassigned progeny. Gerud 2.0 produces a more conservative estimate of the number of sires, as it will attribute these two offspring to one

father. Colony 1.2 will assign them to one or two fathers depending on the likelihood of each outcome (determined by population allele frequencies and the number of shared alleles between progeny). However, the simulations were consistent, allowing us to explore fitness benefits to both sexes in relation to patterns of genetic polyandry and reproductive skew. The direct benefit to multiple mating can be examined by estimating the slope of the least squares regression between reproductive success and number of mates (Bateman 1948; Arnold 1994; Arnold & Duvall 1994; Jones *et al.* 2000). In *C. plumbeus*, these slopes were flat ( $B = 0.43$ ,  $B = 0.98$ ) and not significantly different than zero, suggesting that there may be little direct benefit for multiply inseminated females (Andersson & Iwasa 1996). The slope produced through linear regression results in the best approximation of selection gradients but may not be the best fit for the data (Lande & Arnold 1983). The absolute values of point estimates of slopes were larger for males than females ( $B = 1.12$ ,  $B = 1.30$ ) and significantly different than zero, suggesting males receive direct benefit by limiting the number of additional males gaining access to a female's ova. Since the estimated  $B$  for females varied depending on whether Gerud or Colony results were used and confidence intervals were large, a second measure was used to validate our conclusions. Calculating the standardized variance of reproductive success ( $I$ ) for males and females allows for an estimate of the amount of selective force the sexes are experiencing (Wade 1979; Wade & Arnold 1980). These measures corroborated the above conclusion as males had larger  $I$  values than females, suggesting there is greater opportunity for selection on males to limit the number of additional sires contributing to a litter than there is for females to acquire additional sires. Together, these measures suggest that while there may be little direct benefit to females who mate multiply, the

ability to bias paternity should be selected for in male sandbar sharks. While this study was unable to distinguish whether male *C. plumbeus* bias paternity through pre-copulatory (behavioural) or post-copulatory (physiological) mechanisms and direct observational data on this species reproduction are lacking, it seems likely that intrasexual competition is important in the evolution of male reproductive behaviour in this species.

The widespread genetic polyandry seen in *C. plumbeus*, in the absence of strong direct selection for females to mate multiply, may indicate that genetic benefits promote the maintenance of polyandry. Since these benefits affect an organism's inclusive fitness (reproductive success of offspring) they are difficult to demonstrate, but have been shown in a number of taxa (reptiles, Olsson *et al.* 1996; eutherian mammals, Keil & Sachser 1998; bony fishes, Evans & Magurran 2000; metatherian mammals, Kraaijeveld-Smit *et al.* 2002). In internally gestating animals such as the sandbar shark, the avoidance of genetic incompatibility, often caused by inbreeding (Zeh & Zeh 1997), may be an important genetic benefit for females who mate multiply. Mating in sharks is particularly costly to females due to blood loss caused by male biting (Springer 1960) and from vaginal lesions (Pratt 1979) resulting from the anchor-like morphology of the distal end of the male's splayed intromittent organs. One might expect polyandry to be common in sharks with small population sizes and low dispersal capabilities such as *G. cirratum*, or in sharks that show philopatry to isolated breeding grounds such as *N. brevirostris*. In these sharks, the genetic benefits of inbreeding avoidance may be great enough to outweigh the costs of mating. Conversely, highly dispersive species with larger population sizes may be more likely to breed monogamously because the chances of

inbreeding are lower while the costs of mating are still high (Chapman *et al.* 2004). In the western North Atlantic the sandbar shark has a wide range, large population size, and centralized mating location (Springer 1960); characteristics that would lead to the expectation of monogamy. However, genetic monogamy does not appear to be common.

Increased within-litter genetic variance caused by polyandrous mating may be a more important form of genetic benefit for female *C. plumbeus*. For females with reproductive cycles greater than one year, mating opportunity is limited and polyandrous mating may ensure increased genetic variation in progeny over a lifetime. In serially monogamous species that mate annually, this benefit may not be great enough to outweigh the cost of mating. Female sandbar sharks are believed to require a quiescent period between reproductive efforts, and likely do not mate annually (Springer 1960; Joung & Chen 1995). The same is true of both *G. cirratum* and *N. brevirostris* (Feldheim *et al.* 2002; Pratt & Carrier 2001). Female *S. tiburo*, in which monogamy is common, reproduce annually (Chapman *et al.* 2004). This pattern lends support to the idea that reproductive periodicity may be important in determining the rate of polyandry. The benefit of increased genetic variation across litters, however, affects a female's inclusive fitness. Such indirect benefits are thought to be smaller than direct benefits and therefore may not outweigh mating costs (Cameron *et al.* 2003).

Alternatively, while there may be some form of indirect female benefit, the lack of relationship between female size and number of sires may reflect the inability of female *C. plumbeus* of any size to control mating frequency. When mating is physically costly, genetic benefits may not improve fitness enough to encourage multiple matings beyond the minimum required to ensure the fertilization of all ova (Brown *et al.* 2004;

Maklakov & Lubin 2004).). While sandbar sharks in the western North Atlantic have a one-to-one sex ratio overall, segregation of the sexes results in sex ratios that vary in space and time (Springer 1960; Musick *et al.* 1993; Burgess unpublished data). Females migrate long distances to give birth in nursery grounds such as Chesapeake Bay and Delaware Bay, where adult males are seldom seen. Mating, on the other hand, takes place at centralized mating grounds off the Atlantic coast of Florida. However, because females are thought to reproduce once every two years and males annually, there is likely a male-biased operational sex ratio (OSR) on the mating grounds. The number of attempts by males to force or steal copulations has been shown to increase across taxa as OSR becomes more male biased (Shine *et al.* 2003; Byrne & Roberts 2004; Fitze *et al.* 2005; Head & Brooks 2006). Population densities may also change intersexual contact rates and consequently reproductive behavior (Westneat and Sherman 1997). As the density and persistence of males increases, female resistance may become difficult. In shark species, multiple males have been observed attempting to breed simultaneously or blocking female access to refugia (Carrier *et al.* 1994; Pratt & Carrier 2001).

When the costs associated with resistance outweigh the costs of mating females may engage in convenience polyandry (Thornhill & Alcock 1983) and the level of genetic polyandry may be maintained above the female optima. This dynamic has been previously documented in other taxa (Rowe 1994; Lee and Hays 2004). Since females must cooperate to allow successful copulation, in species like the sandbar shark, female mating rates should be seen as evolving reaction norms, by which females seek to situationally maximize their fitness, rather than fixed optima (Arnqvist & Nilsson 2000). Experimental work with damselflies and guppies demonstrated that females were more

likely to engage in superfluous copulations when the costs associated with resistance were great (Kelly *et al.* 1999; Cordero & Andres 2002). In these situations more aggressive or persistent males may gain additional copulations, while more resistant females are able to avoid superfluous harmful matings. The increase in fitness for both sexes at the phenotypic extremes of aggression and resistance can lead to sexually antagonistic coevolution (Holland & Rice 1998; Chapman *et al.* 2003). The results of such contests are the evolution of secondary characteristics used to ameliorate the costs of mating or involved directly in male aggression or female resistance (Lessells 2006). In elasmobranchs the thick skins of female sharks (Pratt & Carrier 2001), sexual segregation (Klimley 1985), and the seasonal development of mating teeth by males of many Batoids (Kajiura & Tricas 1996) may be examples of such characters. Parallel characters that are seen in insects where sexually antagonistic coevolution is thought to operate include male and female grasping/anti-grasping structures in water striders (Rowe and Arnqvist 2002) and the use of accessory gland products (Chapman *et al.* 1995).

In this study we found high levels of genetic polyandry in western North Atlantic sandbar sharks. Our findings, however, suggest that neither direct female benefits nor avoidance of genetic incompatibility adequately explain the pattern of male fertilizations in our data. Additionally, our data suggest there may be more selective pressure for males to bias paternity than for females to mate multiply, indicative of intense intrasexual competition. While other cryptic genetic benefits for females cannot be discounted, we feel that coercive male mating tactics are likely important in dictating the number of matings in which a female engages. When examining patterns of polyandry in wild

populations it is therefore important to account for intra-masculine competition, as well as differing male and female motivations for reproductive behaviour.

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Table 1: Summary statistics for five microsatellite markers: allele number (A); gene diversity (h) calculated in F-stat; number of individuals screened (N); conformance to HW equilibrium (p(hw)) calculated in Genepop; exclusion probabilities (P(e)) calculated in Gerud.

Locus	Motiff	A	h	N	p(hw)	P(e)
Cpl 90	(AC)24	27	0.930	70	0.45	0.856
Cpl 128	(CA)13TA(CA)13	16	0.870	70	0.66	0.746
Cpl132	(TG)16	12	0.836	71	0.50	0.670
Cpl 166	(GT)17	45	0.972	67	0.90	0.930
Cpl 169	(TG)42	36	0.942	73	0.12	0.870

Table 2: Summary of Gerud and Colony estimates of paternal contribution for *C. plumbeus* litters; minimum number of sires suggested by Gerud (Sires), most likely ratio of paternal contribution obtained from Gerud (Skew), number of Gerud solutions which returned the same paternal contribution ratio (#), total number of Gerud solution (Total), number of additional Gerud solutions with different paternal contribution (Alternative), number of sires suggested by Colony (Sires 2), ratio of paternal contribution obtained from Colony (Colony).

Litter	Location	Size	Sires	Skew	#	Total	Alternative	Sires 2	Colony
A	SA	10	1	NA	1	1	NA	1	NA
B	GOM	6	2	3:3	1	1	NA	2	3:3
C	GOM	10	3	6:2:2	20	20	NA	4	6:2:1:1
D	GOM	12	1	NA	1	1	NA	1	NA
E	SA	9	2	7:2	2	2	NA	2	7:2
F	GOM	9	2	7:2	1	1	NA	3	7:1:1
G	SA	8	2	6:2	1	1	NA	3	6:1:1
H	GOM	10	3	6:2:2	12	12	NA	4	6:2:1:1
I	GOM	4	2	2:2	6	6	NA	3	2:1:1
J	GOM	12	4	5:3:2:2	41	50	1	5	4:3:2:2:1
K	GOM	13	4	5:3:3:2	50	50	NA	4	5:3:3:2
L	GOM	9	2	5:4	2	2	NA	2	5:4
M	SA	10	2	8:2	1	1	NA	2	8:2
N	SA	10	2	7:3	1	1	NA	2	7:3
O	SA	11	3	6:4:1	1	6	3	3	6:4:1
P	SA	7	2	4:3	1	1	NA	2	4:3
Q	GOM	10	3	6:2:2	4	6	1	3	6:3:1
R	GOM	8	1	NA	1	1	NA	1	NA
S	SA	9	3	5:2:2	4	4	NA	4	5:2:1:1
T	SA	10	2	7:3	1	1	NA	2	7:3

Figure 1: Frequency distribution of male reproductive success for males who sired greatest number of progeny in a given litter (DOM) and males who sired remainder of progeny in a given litter (NON). Dashed line is the expected distribution of mating success for all males, if it was determined by random processes (mean reproductive success calculated from data = 4.1). Solid line is the expected distribution of mating success for the most successful males only, if it was determined by random processes (mean reproductive success calculated from data = 6.2)

### Frequency of Male Success

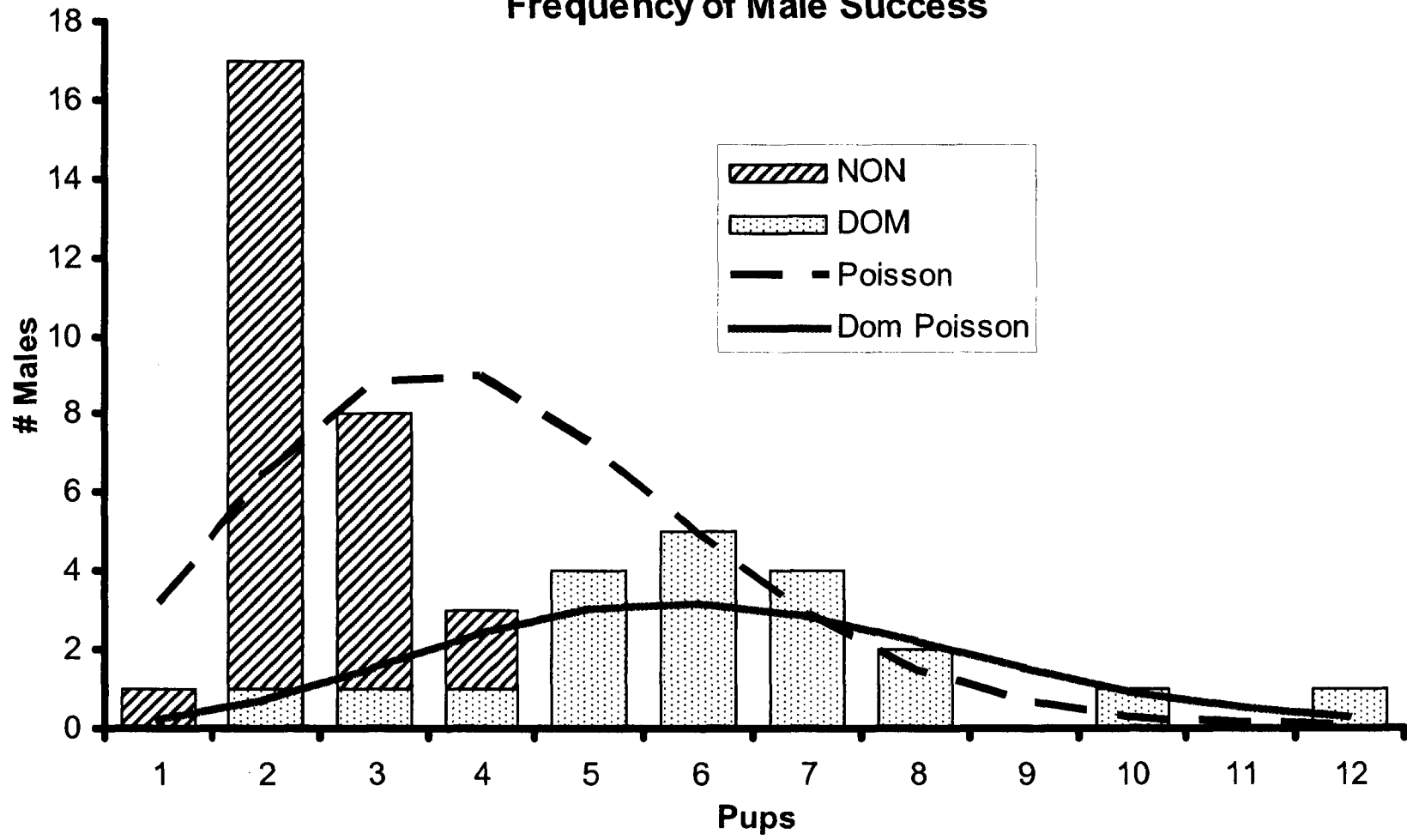
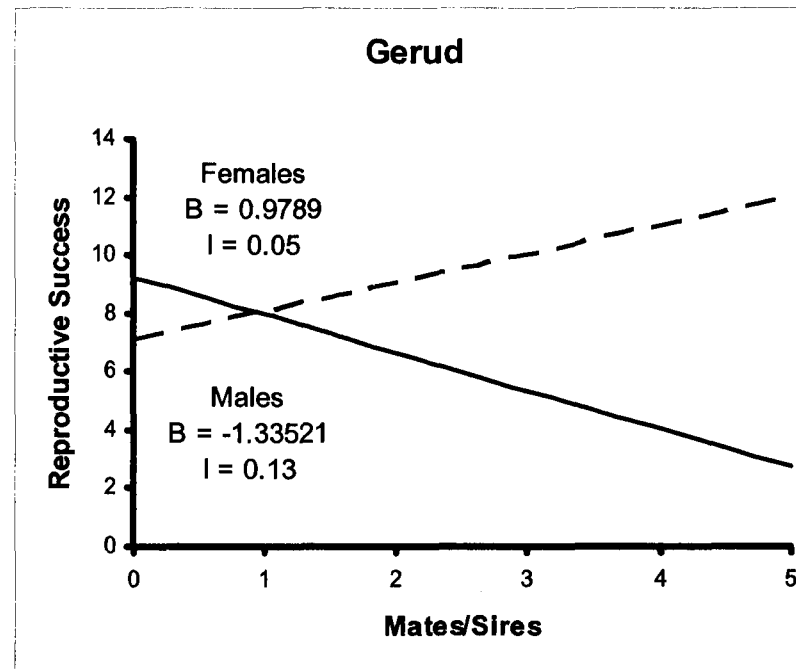
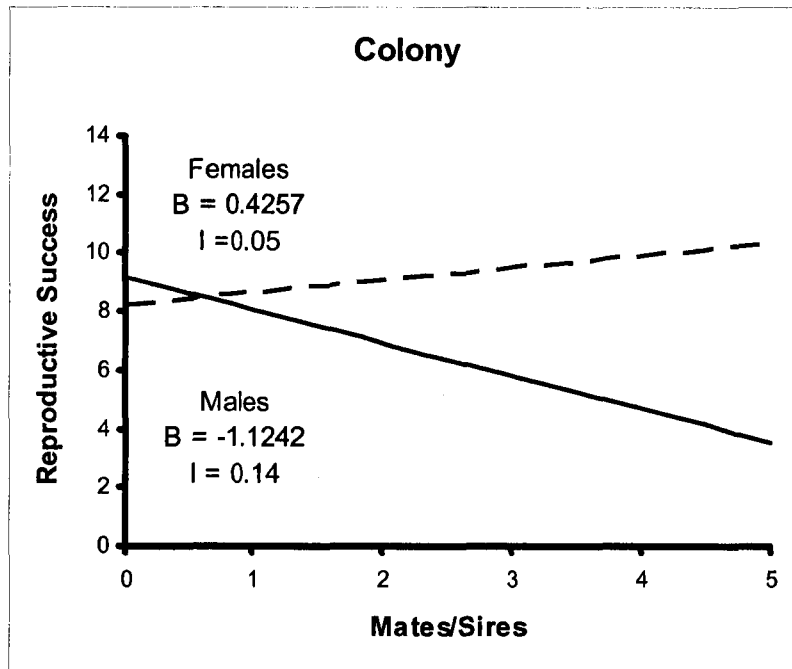


Figure 2: Relationships between reproductive success and number of mates per litter for females (dash line) and reproductive success and number of additional sires for "successful males" (solid line) using Gerud and Colony data. Estimates of the intensity of selection ( $I$ ) support point estimates of the slope ( $B$ ) calculated by least squares regression. In this case the larger  $B$  and  $I$  values for the male data suggest that there may be direct benefit for males that limit the number of additional sires in a litter, while there is no direct benefit for females who are multiply inseminated.



### Chapter 3

Effective Number of Breeders Closely Approximates the Census Size in the  
Heavily Exploited Western North Atlantic Population of Sandbar Sharks,  
*Carcharhinus plumbeus*

**Abstract:**

The sandbar shark, *Carcharhinus plumbeus*, is a heavily fished species throughout much of its range including in the western North Atlantic. Like most elasmobranchs it is long-lived and has low lifetime fecundity. Inshore nursery grounds serve to increase survivorship of sandbar shark pups and juveniles at a time when they are most vulnerable to predation, and the most important nursery grounds are in the mid-Atlantic region. We calculated the effective number of breeders ( $N_b$ ) and effective population ( $N_e$ ) size for adults utilizing two of these nursery grounds, Delaware Bay and the lagoons of the Eastern Shore of Virginia, by genotyping 902 animals across five cohorts (2002-2006) at eight polymorphic microsatellite loci. Effective size estimates were then compared to estimates of census size ( $N_c$ ) of the 2004, 2005 and 2006 cohorts obtained from Delaware Bay. The  $N_e/N_c$  ratio was 0.45 or higher whether the Delaware Bay cohorts were considered as distinct year classes or combined. This finding is in sharp contrast to the  $N_e/N_c$  ratios found in other exploited marine species, which are usually several orders of magnitude smaller. Instead the  $N_e/N_c$  ratio of sandbar sharks is similar to that found in many marine and terrestrial mammals. The close coupling of census and effective size observed in the sandbar shark suggests that intense fishing may have a more direct detrimental impact on adaptive genetic variance in this and other shark species than it does in bony fishes.

## Introduction:

Effective size ( $N_e$ ) is an important consideration for wildlife conservation and management because it is inversely proportional to the rate at which drift and inbreeding alter genetic variance (Wright 1931). Populations with small  $N_e$  are more susceptible to the fixation of deleterious alleles and loss of additive adaptive variance, evolutionary changes that may lead to extirpation (Franklin 1980, Frankham 1996, Newman and Pilson 1997). There is no direct relationship between  $N_e$  and census size ( $N_c$ ). The ratio of the two measures varies greatly, from  $10^{-5}$  in many marine species to nearly 1.0 in some terrestrial vertebrates (Frankham 1995, Hedrick 2005), and therefore,  $N_e$  must be estimated from demographic and/or genetic data. Difficulty in obtaining the information required for demographic methods of estimating  $N_e$  has led to multiple formulations for estimating  $N_e$  from genetic data (Caballero 1994, Wang 2005).

There are two major categories of  $N_e$  estimates, contemporary  $N_e$  and historic  $N_e$ . While the latter has been examined in a conservation context by several authors (Roman and Palumbi 2003, Alter *et al.* 2007), it must be interpreted carefully as past demographic change has great affect on the estimate (Crandall *et al.* 1999, Schwartz *et al.* 1999). This makes the results less informative for those interested in a current population's evolutionary potential. On the other hand contemporary estimates of  $N_e$  apply to generations in the recent past and estimates of the effective number of breeders ( $N_b$ ) apply directly to the parents of a sampled cohort (Waples 2005). Thus, these measures are more useful for proactive conservation and management.

In recent years, there has been increased interest in the incorporation of  $N_e$  estimates in fisheries management and conservation (Ryman *et al.* 1995, Ashley *et al.* 2003). In particular, there is concern that fishing may not only act as a selective agent (Law 2000), but may simultaneously reduce genetic variance (Pichler and Baker 2000, Jones *et al.* 2001, Hauser *et al.* 2002, Hutchinson *et al.* 2003). Estimation of contemporary  $N_e$  in this context has mostly relied on various versions of the temporal method; in which  $N_e$  is estimated from the variance in allele frequencies between two samples separated in time. To produce accurate estimates these methods generally require samples at least one generation apart (Waples 1989, Williamson and Slatkin 1999, Wang 2001). In fact, this method has been widely used in bony fishes (Hauser *et al.* 2002, Hutchinson *et al.* 2003, Hoarau *et al.* 2005, Poulsen *et al.* 2006) via archived scales or otoliths collected for aging studies. To date, there has not been an assessment of current effective size for any shark species. This may be in part due to the lack of archived materials, as shark scales are not used in aging studies, but also because most shark fisheries have generally existed over periods of time that are short relative to the target species' generation times (Anderson 1990, Hoff and Musick 1990). Thus to estimate  $N_e$  either methods that require single samples (Waples 1991) or modified temporal estimators (Jorde and Ryman 1995) are more appropriate for use with elasmobranchs.

Estimating current  $N_e$  for elasmobranchs is important because many species are fully exploited or overexploited in fisheries through out the world's oceans and historically have not fared well under fishing pressure (Ripley 1946, Olsen 1959, Aeson 1964, Musick *et al.* 2000). In addition elasmobranchs share life history characteristics

such as slow growth, late maturity, internal gestation and low fecundity with mammals (Walker 1998, Stevens *et al.* 2000) and other characteristics, such as high dispersal potential, with bony fishes. Since the  $N_e/N_c$  is several orders of magnitude larger in terrestrial vertebrates than marine fishes (Frankham 1995, Hoarau *et al.* 2005) and elasmobranchs have a distinct evolutionary lineage basal to other vertebrates, understanding the relationship between  $N_e$  and  $N_c$  in elasmobranchs will be evolutionarily informative as well.

The sandbar shark, *Carcharhinus plumbeus*, is a heavily exploited species throughout most of its global range (McAuley *et al.* 2007). The species reaches maturity slowly and has low lifetime fecundity (Sminkey and Musick 1996), making it vulnerable to over-exploitation. The western North Atlantic population encompasses animals caught from Cape Cod all the way to the Gulf coast (Heist *et al.* 1995). In the western North Atlantic the sandbar shark comprises more than 2/3 of the directed commercial shark fishery (Castro 1993), and the stock has been in decline since the inception of the fishery (Musick *et al.* 1993). Mating occurs off the Atlantic coast of Florida and females, who bear live young, make long migrations in the summer every other year to inshore nursery grounds to give birth (Springer 1960). Nursery grounds are vital to the species as they provide both an abundant supply of food for the growing pups and safety from large elasmobranch predators found in greater number to the south (Springer 1967, Medved *et al.* 1985). Juveniles move offshore in the winter months but return to their natal nursery every summer for the first 3-14 years of life (Grubbs *et al.* 2007, McCandless *et al.* 2007).

The most important western North Atlantic nursery grounds are thought to be in the mid-Atlantic and include the lower Chesapeake Bay, the lagoons of the Eastern Shore of Virginia, and Delaware Bay (Grubbs and Musick 2007, McCandless *et al.* 2007). Therefore, estimating  $N_b$  and  $N_e$  in the Delaware Bay (DEL) and Eastern Shore Lagoons (ES) may provide critical information about the long-term sustainability of the Atlantic stock. While temporally spaced samples are not available for such an estimate, as generation time is around 20 years, sampling in the summer allows for the collection of samples from discrete cohorts. This in turn allowed us to estimate  $N_b$  and  $N_e$  using the linkage disequilibrium method (Hill 1981, Waples 1991 Waples 2006) as well as a modified temporal method (Jorde and Ryman 1995) in two of the vital nursery areas and compare the values with estimates of census size.

#### Materials and Methods:

##### Collection and Genotyping

Juvenile sharks were captured from seaside lagoons on the Eastern Shore of Virginia and from within the Delaware Bay between May and September 2003-2006 using research longline and gillnet gear as described by Branstetter and Musick (1993) and McCandless *et al.* (2007). Total length, fork length and standard length (length from tip of the snout to just before the caudal fin) were measured for each fish. A small piece of tissue was excised from the trailing portion of the first or second dorsal fin and animals were released. Tissue was stored in 10% DMSO buffer (Seutin *et al.* 1991) at 4° C till extraction.

Since sandbar sharks exhibit placental viviparity, newborns have the remnants of the umbilicus and young of the year (YOY) animals retain obvious umbilical scarring throughout the first months after birth. Therefore, individuals with open or recently healed birth scars were considered to be YOY. Since juveniles return to their natal nursery grounds, older individuals were used to augment cohorts where there were few YOY samples. As age regressions lack accuracy, because juvenile sharks were captured in this study at the time of the year when growth is greatest (Grubbs *et al.* 2007), model progression analysis, following the methodology of Bhattacharya (1967), was implemented in FiSAT II (Gayanilo *et al.* 2005) to determine the age of older animals or those with late stage birth scars. Briefly, size distribution data for each month across sampling years was pooled and used to create a plot of log frequency difference against midpoint length. Regression lines were created which defined the first two moments of the Gaussian distributions. The slope of this regression is indicative of the variance and the x intercept is the median of the distribution. All individuals within 2.0 standard deviations of the mean size for an age class were defined as belonging to that cohort. In later months, when the distributions show greater overlap as variance in growth rate increases, individuals within 1.5 standard deviations of the mean size for an age class were defined as belonging to that age class.

DNA was extracted using a modified Chelex extraction protocol (Estoup *et al.* 1996). After a two minute centrifugation at 16,000g, 0.3ul of the supernatant was used directly as a template for PCR reactions. A total of 902 juvenile sandbar sharks were genotyped at eight microsatellite loci. Markers were amplified for each individual using IR-700 and IRD-800 labelled forward primers. Descriptions of primers and PCR

conditions for the six species-specific markers, Cpl153, Cpl190, Cpl128, Cpl132, Cpl166, Cpl169 are reported elsewhere (Portnoy *et al.* 2006, Portnoy *et al.* 2007) Two additional markers, Cli12 and Cli103, originally isolated from the congeneric blacktip shark, *Carcharhinus limbatus*, were surveyed following the protocols outlined in Keeney and Heist (2003). All amplicons were electrophoresed through 25 cm 6.5 % polyacrylamide gels using a LiCor 4200 Global IR<sup>2</sup> system. A 50-350 bp size standard was run in the first, middle, and last lanes of each gel and locus-specific standards were run in every 4th lane. Alleles were scored manually with the aid of Gene ImagIR 4.05 (Scanalytics, Rockville MD). Twenty-five percent of samples were randomly selected and rescored to ensure accurate scoring. Individuals for which more than two loci could not be reproducibly scored were discarded.

#### Genetic Data Analysis

Conformance to the expectations of Hardy-Weinberg equilibrium was calculated in GENEPOP (Raymond & Rousset 1995) for each locus using 93-96 individuals selected to be representative of the species throughout its range in the western North Atlantic and Gulf of Mexico. Exact tests were run with 10,000 iterations. Number of alleles and allelic diversity were calculated for each locus for the combined data set of juvenile samples using FSTAT (Goudet 2001). Micro-Checker (Oosterhout *et al.* 2004) was used to screen for null alleles and genotyping errors.  $N_b$  and  $N_e$  were calculated using the linkage disequilibrium method in the program LDNE (Waples and Do 2008). In brief, this methodology calculates the correlation among alleles at unlinked loci ( $r$ ),

which can be related to  $N_e$  by the formula  $N_e = \frac{1}{3 * (r^2 - \frac{1}{S})}$  (Hill 1981, Waples 1991),

where  $S$  is sample size. To correct for downward bias associated with small sample sizes, LDNE uses a modified version of this equation (Waples *et al.* 2006). The data were analyzed both keeping cohorts within nurseries separate and with all cohorts combined within nurseries. Analyses were run sequentially excluding minor alleles at the 0.01, 0.02 and 0.05 frequency levels.

$N_e$  was also estimated for ES and DEL samples using a modified temporal method (Jorde and Ryman 1995). In brief, this method examines shifts in allele frequencies between consecutive cohorts and relates them to  $N_e$  by the formula  $N_e = \frac{C}{2GF_k'}$ , where  $G$  is generation time,  $F_k'$  is Pollack's F-statistic averaged across cohorts, and  $C$  is a parameter used to account for the probability of survival to age ( $l_i$ ) and reproductive output of each age class ( $b_i$ ).  $F_k'$  was calculated between consecutive cohorts sequentially excluding minor alleles at the 0.01, 0.02 and 0.05 frequency levels using SalmonNb (Waples *et al.* 2007) and then averaged. In order to estimate  $C$  and  $G$ , values of  $l_i$  were calculated using mean age-specific survivorships (Cortes and Brooks 2005). Given that there is no detectable relationship between female size and reproductive output in sandbar sharks and males are not likely sperm limited (Portnoy *et al.* 2007),  $b_i$  was calculated from the proportion mature individuals in each age class using two different maturity ogives (Merson 1998, Romine unpublished data), and  $G$  and  $C$  were calculated on a windows executable program (P. Jorde, personal communication). Confidence intervals

were calculated assuming that the F-statistic is chi-square distributed (Waples 1989, Jorde and Ryman 1996).

Census estimates for the number of breeders were generated for DEL 2004, 2005 and 2006. Briefly, the number of YOY sharks in the estuary (McCandless unpublished data) was divided by 8.4, the average yearly reproductive success of females (Sminkey and Musick 1996), to arrive at an estimate of the number of mature females. To arrive at an estimate of census size the estimated number of females was then multiplied by 3.3, to account for the average number of sires per litter (2.3, Portnoy *et al.* 2007). A more conservative estimate of census size was also made by multiplying the number of females by 2 to account for the adult sex ratio which is 1:1 (Springer 1960). Estimates of  $N_e$  made excluding alleles at frequencies less than 0.02 were then compared with census size. This exclusion category of estimates was used because they are conservative enough to eliminate noise created by highly variable loci but retain some of the information they provide (R. Waples personal communication). For this reason they will also be the focus of the results and discussion section unless otherwise specified.

#### Results:

The number of alleles per locus varied from 6 at Cpl 53 to 74 at Cpl 166. The genotypic distributions of all loci conformed to the expectations of Hardy-Weinberg equilibrium and no evidence of null alleles or scoring error due to stutter-bands was detected at any locus using the Micro-checker software. Since the methodologies used in this study exclude low frequency alleles, null alleles are unlikely to affect the estimates of

$N_e$ . All summary statistics, as well as expected and observed numbers of heterozygotes, are available in electronic Appendix A.

The linkage disequilibrium method returned fairly consistent estimates of  $N_b$  within nurseries across years. For most years, at least one estimate had confidence intervals that did not include infinity and estimates were often consistent across minor exclusion categories (0.01, 0.02 and 0.05, Table 1). For the most part, estimates were smaller with higher exclusion frequencies and larger with lower exclusion frequencies (Table 1). Yearly estimates of  $N_b$  were larger in Delaware Bay than in the Eastern Shore Lagoons (Table 1), with the harmonic means of 1059 and 511, respectively. When the data were summed across years within nursery grounds, the linkage disequilibrium estimate of  $N_e$  was 4890 (760.5 -  $\infty$  at 95% CI) for DEL and 2709 (1451.9 - 13792.9 at 95% CI) for ES (Table 2).

Generation time calculated for use with the Jorde and Ryman method was 20.88 years when the Merson (1998) ogive was used and 19.04 when the Romine ogive (J. Romine personal communication) was used. The C parameter estimates based on the different ogives were 69.549 and 71.509 respectively, and both stabilized after about 100 generations (life history tables used to calculate G and C are available as electronic Appendix B). Though both sets of parameters gave similar  $N_e$  estimates, those using the Romine ogive were consistently larger (Table 2) and will be considered in the following results and discussion. Estimates of  $N_e$  using the Jorde and Ryman method for ES were consistent when minor alleles were excluded at the 0.01 and 0.02 levels, 1619 (1325.5-1719.0 at 95% CI) and 1409 (1152.6 - 1687.0 at 95% CI), respectively. However, when alleles less frequent than 0.05 were excluded, the estimate was somewhat larger; 3954

(3266.3-4708.0). Estimates of  $N_e$  for DEL were less consistent with estimates at the 0.01 and 0.05 exclusion level being 2974.9 (2480.7-3509.356) and 1177.2 (961.7-1412.4). An estimate could not be made at the 0.02 level because sampling error was too great compared to  $F_k$  between 2005 and 2006.

The census number of age zero sharks in Delaware Bay was estimated at 5826 in 2004, 6006 in 2005, and 4474 in 2006 (McCandless unpublished data). This corresponds to approximately 693, 715, and 533 mature females and a census number of breeders of 2289, 2360 and 1758 when patterns of polyandry are taken into account or 1387, 1430 and 1065 when only the sex ratio is accounted for.  $N_b/N_c$  was 0.45 or 0.75 in 2004, 0.46 or 0.75 in 2005 and 0.57 or 0.94 in 2006 (Table 1). Using the linkage disequilibrium method over the three year period  $N_e/N_c$  was 0.76 or greater than one. The smallest  $N_e$  estimate using this methodology, at the 0.05 level, yielded ratios of 0.51 or 0.84. Using the Jorde and Ryman temporal method  $N_e/N_c$  was 0.56 or 0.92 at 0.05 level but was greater than one at the 0.01 level (Table 2).

#### Discussion:

We were able to obtain robust estimates of both  $N_e$  and  $N_b$  with reasonable sample sizes using both methods, making these approaches useful for conservation and management of shark species. Our estimates varied slightly between methods and exclusion categories, but were of the same magnitude. Low sample size for DEL06 ( $N=53$ ) affected estimates of  $r^2$ , resulting in problems when using the linkage disequilibrium method to estimate  $N_b$ . This is not surprising as multiple authors have

noted the inaccuracy of the method when  $N/N_e < 0.1$  (England *et al.* 2006, Waples 2006). In addition, the small sample size of DEL06 likely caused the observed inconsistencies in the estimates of  $N_e$  using the Jorde and Ryman method, as it affected the estimate of  $F_k$  between 2005 and 2006. For the remainder of samples, however, the methodology worked well with reasonable sampling effort ( $N=77-139$ ). A concern was that the inclusion of animals collected up to two years after birth might affect estimates, if animals stray from their natal nursery. Yet the ES02 estimate, which was composed entirely of tissues collected in 2003 and 2004, was consistent with all other years. In fact, if juvenile straying had been present, one would expect the estimate of  $N_b$  would be larger for that year. However, it was the second smallest estimate. Finally, since for females, parturition occurs only once in a given year, estimates within the same year at the different nurseries should be considered independent. Caution should be taken in summing these estimates within a year across sampling locations to get cumulative reproductive effort estimates because males are likely represented in progeny found in both locales.

The ratios of  $N_b/N_c$  and  $N_e/N_c$  in sandbar sharks were found to be close to 0.5 which conforms to expectations for random mating populations with overlapping generations (Nunney 1993). It is important to note that comparisons of  $N_e/N_c$  across studies must be made with caution not only because methodologies for calculating  $N_e$  differ, but because the appropriate definition of  $N_c$  will differ as well (Nunney and Elam 1992). Even within this study, the different methods of estimation yielded slightly different  $N_e/N_c$  ratios, because  $N_e$  and  $N_c$  are calculated as harmonic means across years in the Jorde and Ryman temporal method, whereas they are point estimates in the linkage

disequilibrium method (Waples 2005). In addition, as  $N_c$  decreases, variance in reproductive success may decrease causing an increase in  $N_e/N_c$  (Arden and Kapuscinski 2003). Nonetheless, our estimates of  $N_e/N_c$  were similar and the smallest ratio obtained in this study (0.45) was higher than the average for wildlife of 0.10-0.11 reported by Frankham (1995) and orders of magnitude larger than most marine species examined  $10^{-3}$ - $10^{-5}$  (Hoarau *et al.* 2005).

The relatively high  $N_e/N_c$  seen in sandbar sharks is much closer to values reported for mammals than for marine fishes (Figure 1). Variation of family size, unequal contribution of males and females to breeding, and non-random mating are all factors that cause effective size to be lower than census size (Falconer and Mackay 1996). In marine species, which are typically highly fecund, the low ratio has been attributed to large variance in reproductive success (Hedgecock 1994). Female sandbar sharks invest heavily in decreasing offspring mortality through long gestation periods and migrations to nursery grounds (Branstetter 1990), and there is low variance in female fecundity (Sminkey and Musick 1996). In addition, an even sex ratio (Springer 1990), and aggressive male mating tactics that may make female mate choice difficult (Pratt and Carrier 2001, Portnoy *et al.* 2007) are factors that could maintain  $N_e$  close to  $N_c$ . Many of these characteristics are shared by other shark species and some, such as increased parental investment and increased offspring survival, are also present in mammals.

The close coupling of  $N_e$  and  $N_c$  in the sandbar shark in the face of exploitation may be cause for concern. Populations with  $N_e$  smaller than 500 are thought to be at risk of losing genetic variation via drift (Franklin and Frankham 1998). Our estimates of  $N_b$  were on the order of 400-1000 and  $N_e$  estimates made across year were at least twice as

large (1408-4890). However, there is evidence that the  $N_e$  needed for a population to retain evolutionary potential may be as large as 5000 (Nunney and Campbell 1993, Lande 1995). Furthermore, marine species with low  $N_e/N_c$  ratios tend to feature high fecundity and/or population growth rates. These species may have the potential to maintain genetic diversity and/or avoid the fixation of deleterious alleles despite large fluctuations in  $N_c$  (Lesica and Allendorf 1992, Mills and Smouse 1994, Lynch *et al.* 1995). Evidence that populations may be maintained over long periods of varying  $N_c$  with stable  $N_e$  exists for bony fishes (Grant and Bowen 1988, Ruzzante 2001, Poulsen *et al.* 2006). For *C. plumbeus* and other shark species where  $N_e/N_c$  is high and rebound potential is low (Smith 1998), continued removal of biomass may be accompanied by the removal of genetic variance with no means of compensation. While loss of additive genetic variance may not immediately affect fitness, such decreases may leave populations unable to adapt to ecological change, increasing the probability of localized extirpation.

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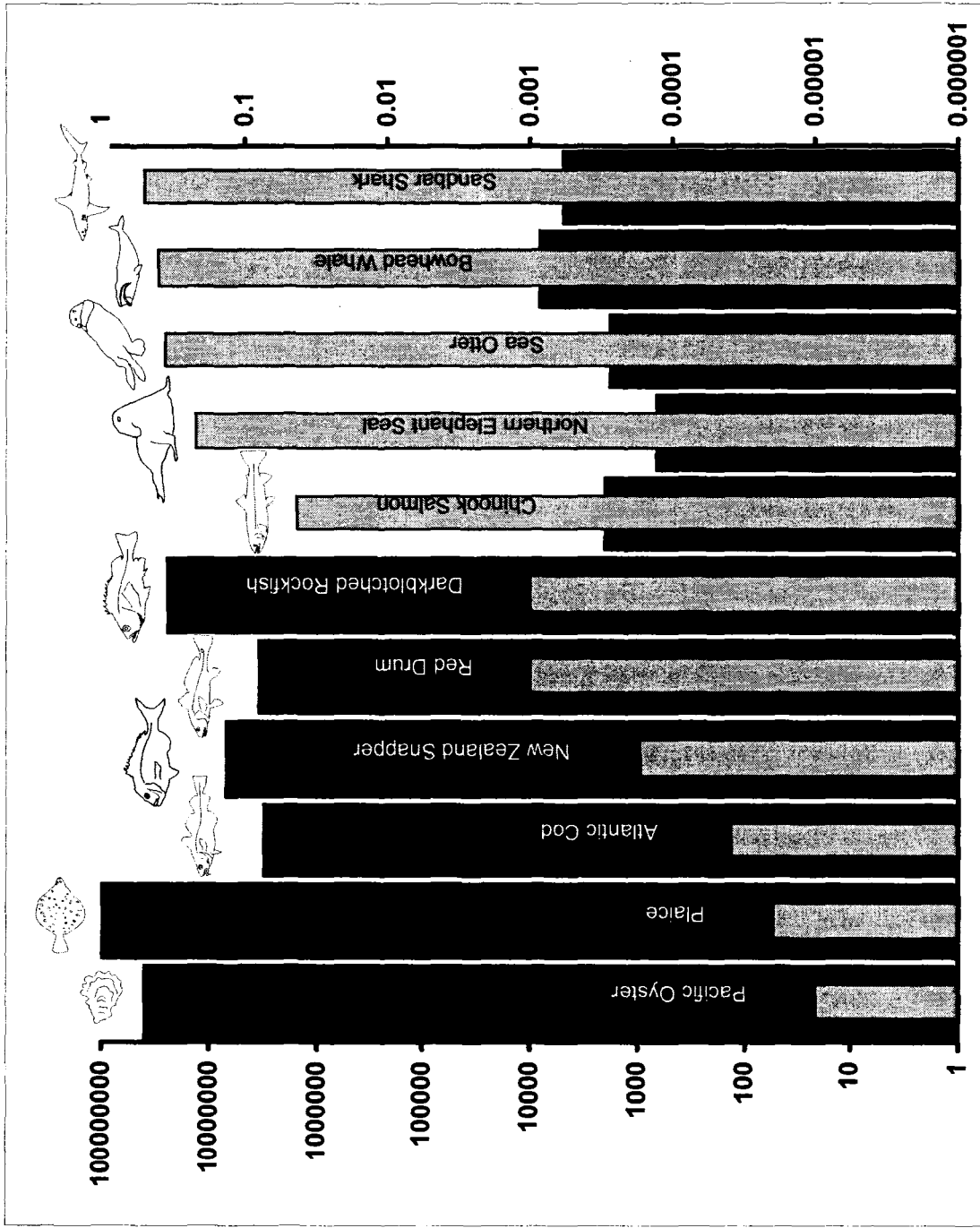
Table 1: Yearly estimates of effective number of breeders ( $N_b$ ) for the lagoons of the Eastern Shore of Virginia (ES) and Delaware Bay (DEL). Estimates were made excluding alleles with frequencies less than 0.01, 0.02 and 0.05.  $N_b/N_c$  was calculated for DEL where two different census size estimates ( $N_c$ ) were available.  $N_b$  values used in the ratio were at the  $<0.02$  level.

cohort	n	$<0.01$	$<0.02$	$<0.05$	$N_b/N_c$
ES2002	77	3751 (567.0- $\alpha$ )	427 (202.1- $\alpha$ )	184 (94.4-919.8)	NA
ES2003	139	1526 (683.9- $\alpha$ )	734 (404.1-3035.9)	886 (256.8- $\alpha$ )	NA
ES2004	99	922 (446.8- $\alpha$ )	469 (267.1-1560.1)	220 (110.6-1208.9)	NA
ES2005	106	1785 (555.7- $\alpha$ )	416 (233.8-1430.4)	227 (120.9-870.8)	NA
ES2006	85	776 (379.8-50295.0)	798 (314.4- $\alpha$ )	276 (114.5- $\alpha$ )	NA
DEL2004	142	1128 (590.6-8051.2)	1038 (487.9- $\alpha$ )	NA	0.45 (0.75)
DEL2005	201	1797 (878.3-154838.5)	1079 (585.1-4985.0)	701 (292.9- $\alpha$ )	0.46 (0.75)
DEL2006	53	3458 (343.8- $\alpha$ )	NA	1000 (113.3- $\alpha$ )	0.57 (0.94)

Table 2: Estimates of effective size ( $N_e$ ) for lagoons of the Eastern Shore of Virginia (ES) and Delaware Bay (DEL) using the linkage disequilibrium method (LD) and the Jorde and Ryman (1995) temporal method (JR). Estimates were made excluding alleles with frequencies less than 0.01, 0.02 and 0.05. Demographic parameters for JD were taken from one of two maturity ogives Romine (unpublished data) or Merson (1998).  $N_e/N_c$  was calculated for DEL where two census size estimates ( $N_c$ ) were available.  $N_e$  values used in the ratio were at the  $<0.05$  level.

	n	<0.01	<0.02	<0.05	$N_e/N_c$
ES(LD)	506	3003 (1762.5-8982.9)	2709 (1451.9-13792.9)	1530 (668.5- $\infty$ )	NA
DEL (LD)	396	3977 (1899.6- $\infty$ )	4890 (1771.4- $\infty$ )	3259 (760.5- $\infty$ )	0.51 (0.84)
ES(JR)Merson	506	1436 (1175.6-1719.0)	1249 (1022.2-1497.1)	3507 (2896.8-4176.3)	NA
DEL (JR)Merson	396	2639 (2201.5-3114.4)	NA	1044 (853.5-1253.4)	0.50 (0.82)
ES(JR)Romine	506	1619 (1325.5-1938.3)	1409 (1152.6-1687.0)	3954 (3266.3 - 4708.0)	NA
DEL(JR)Romine	396	2974 (2480.7.5-3509.4)	NA	1177 (961.7-1412.4)	0.56 (0.92)

Figure 1: Ratio of effective size to census size ( $N_e/N_c$ ) and census size ( $N_c$ ) for wild populations of marine and anadromous species of management and conservation interest (both axes in log-scale). Forward bars are  $N_e/N_c$  back set bars are  $N_c$ . Estimates were taken from the literature (Ralls *et al.* 1983, Bartley *et al.* 1992, Nunney 1993, Hedgecock 1994, Shelden *et al.* 2001, Turner *et al.* 2002, Hauser *et al.* 2002, Hutchinson *et al.* 2003 Hoarau *et al.* 2005, Gomez-Uchida and Banks 2006)



## Electronic Appendix A

Table 1: Summary statistics for eight microsatellite markers, conformance to Hardy Weinberg equilibrium (P is P-value), observed (Ho) and expected heterozygosity (He) calculated using adults sampled from throughout the species range in the western North Atlantic and Gulf of Mexico. Number of alleles (A) and allelic diversity (h) calculated using all juveniles collected in both nurseries.

<b>Locus</b>	<b>Ho</b>	<b>He</b>	<b>P</b>	<b>A</b>	<b>h</b>
<b>Cli12</b>	77	82	0.0977	16	0.850
<b>Cli103</b>	69	66	0.2880	13	0.638
<b>Cpl53</b>	50	52	0.8697	6	0.562
<b>Cpl90</b>	88	88	0.4129	30	0.916
<b>Cpl128</b>	79	80	0.7842	24	0.86
<b>Cpl132</b>	73	78	0.4518	17	0.836
<b>Cpl166</b>	90	91	0.2205	74	0.976
<b>Cpl169</b>	84	87	0.0601	52	0.953

## Electronic Appendix B

Table 1: Life history tables used to calculate C and G for use with Jorde and Ryman temporal method. S mean age-specific survivorship from Cortes and Brooks (2005), L is probability of survival to age, BI is reproductive output for given age class taken from Merson (1983) and Romine (personal communication).

age	S	LI	Merson (1983)	Romine
			BI	BI
0	0.79	1.000000000	0.000	0.000
1	0.83	0.790000000	0.000	0.000
2	0.84	0.655700000	0.000	0.000
3	0.85	0.550788000	0.000	0.000
4	0.86	0.468169800	0.000	0.000
5	0.86	0.402626028	0.000	0.001
6	0.87	0.346258384	0.000	0.002
7	0.87	0.301244794	0.000	0.006
8	0.87	0.262082971	0.000	0.015
9	0.87	0.228012185	0.000	0.038
10	0.88	0.198370601	0.000	0.091
11	0.88	0.174566129	0.000	0.201
12	0.88	0.153618193	0.010	0.390
13	0.88	0.135184010	0.150	0.618
14	0.88	0.118961929	0.350	0.804
15	0.88	0.104686497	0.650	0.912
16	0.89	0.092124118	0.800	0.963
17	0.89	0.081990465	0.850	0.985
18	0.89	0.072971514	0.950	0.994
19	0.89	0.064944647	0.990	0.998
20	0.89	0.057800736	1.000	0.999
21	0.89	0.051442655	1.000	1.000
22	0.89	0.045783963	1.000	1.000
23	0.89	0.040747727	1.000	1.000
24	0.89	0.036265477	1.000	1.000
25	0.89	0.032276275	1.000	1.000

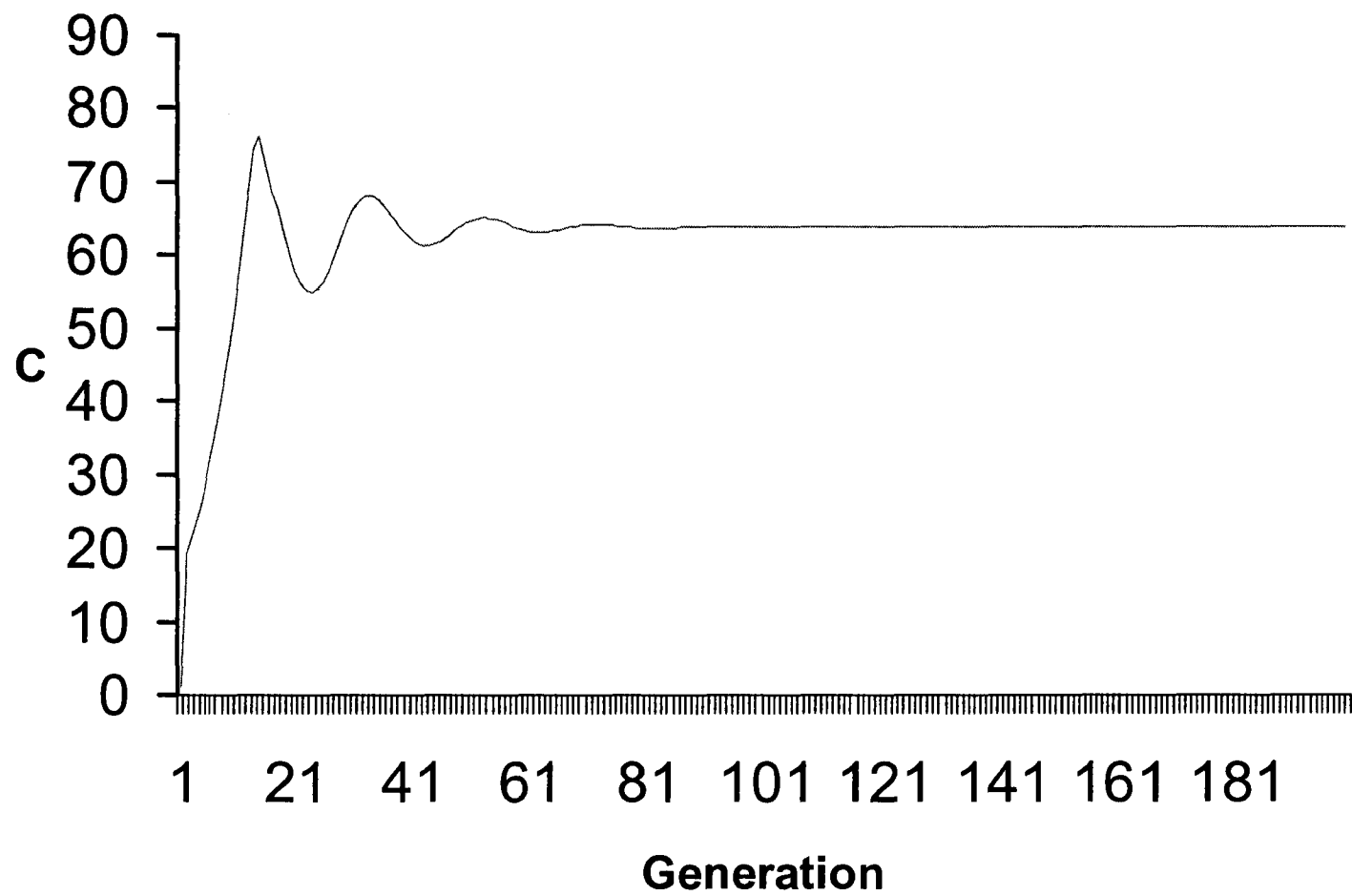
## Electronic Appendix B

Table 1 cont.

<b>age</b>	<b>S</b>	<b>LI</b>	<b>Merson (1983) BI</b>	<b>Romine BI</b>
26	0.89	0.028725884	1.000	1.000
27	0.89	0.025566037	1.000	1.000
28	0.89	0.022753773	1.000	1.000
29	0.89	0.020250858	1.000	1.000
30	0.89	0.018023264	1.000	1.000
31	0.89	0.016040705	1.000	1.000
32	0.89	0.014276227	1.000	1.000
33	0.89	0.012705842	1.000	1.000
34	0.89	0.011308199	1.000	1.000
35	0.89	0.010064298	1.000	1.000
36	0.89	0.008957225	1.000	1.000
37	0.89	0.00797193	1.000	1.000
38	0.89	0.007095018	1.000	1.000
39	0.89	0.006314566	1.000	1.000
40	0.89	0.005619964	1.000	1.000
41	0.89	0.005001768	1.000	1.000
42	0.89	0.004451573	1.000	1.000
43	0.89	0.003961900	1.000	1.000
44	0.89	0.003526091	1.000	1.000
45	0.89	0.003138221	1.000	1.000
46	0.89	0.002793017	1.000	1.000
47	0.89	0.002485785	1.000	1.000
48	0.89	0.002212349	1.000	1.000
49	0.89	0.00196899	1.000	1.000
50	0.89	0.001752401	1.000	1.000

### Electronic Appendix B

Figure 1: Convergence of C parameter, used in Jorde and Ryman Temporal method, on estimated value over 200 generations.



## Chapter 4

### Philopatry and Reproductive Periodicity in the Sandbar Shark, *Carcharhinus plumbeus*

**Abstract:**

The sandbar shark, *Carcharhinus plumbeus*, is a heavily fished species of management concern in the western North Atlantic. It uses nursery grounds in the United States mid-Atlantic region as nursery areas for parturition. Females may be philopatric and have a two year reproductive cycle, but the level of fidelity to nursery grounds and the regularity of the reproductive cycle have not been verified. To this end, genetic data comprised of microsatellite genotypes and mitochondrial control region sequences were analyzed to look for patterns consistent with female philopatry to the Delaware Bay (DEL), Eastern Shore Lagoons of Virginia (ES) and Chesapeake Bay (CB). In addition, the program Colony 1.2 was used to identify kin groups within and between ES and CB to look for patterns consistent with philopatry and to evaluate female reproductive periodicity. Neither analysis detected evidence of strict female philopatry suggesting either that straying is common or that these analyses lacked sufficient power to reveal such behavior if it exists. Furthermore, the data do not support a strict two year reproductive periodicity. While this finding may, in part, be caused by a lack of power in the analysis, it does call for a reevaluation of female reproductive periodicity in the sandbar shark.

## Introduction:

An increasing amount of evidence suggests that female philopatry to nursery grounds is an important behavior found in many sharks (Pratt and Carrier 2001, Heuter *et al* 2004.). This behavior likely increases survivability of YOY and juvenile sharks by insuring that they are born in environments with suitable prey densities, while at the same time providing a safe haven for pups from larger elasmobranch predators (Branstetter 1990). Since strongly philopatric animals may be at greater risk of localized extinction when exploited, defining the presence and fidelity of philopatry for a given species is of great importance (Heuter 1998).

Two different methodologies have been used to detect the presence of female philopatric behavior in sharks. The first utilizes comparisons of genetic data from nuclear and mitochondrial loci. Since mtDNA is maternally inherited and nuclear loci are biparentally inherited, differences in population structure inferred from these two marker types may be used to infer female philopatry and male mediated gene flow. This approach has been used to infer philopatric behavior of females in marine mammals and sea turtles (Palumbi and Baker 1994, Karl *et al.* 1992) as well as with several shark species. In white sharks, *Carcharodon carcharias*, and shortfin mako sharks, *Isurus oxyrinchus*, mtDNA data have shown population structure across ocean basins while microsatellite data have not, indicating regional female philopatry (Pardini *et al.* 2001, Schrey and Heist 2003). At a finer scale, neonate blacktip sharks, *Carcharhinus limbatus*, captured in nursery grounds showed significant difference in mtDNA haplotypic frequencies between collections taken across the Gulf of Mexico, U.S.

Atlantic and Caribbean Sea, while microsatellites allele frequencies were homogenous across locations (Keeney *et al.* 2005). This again indicates possible female philopatry.

While comparisons of nuclear and mitochondrial data have been informative, they have only been able to detect female philopatry to large regions; detecting philopatry to specific nursery grounds requires a different set of techniques. Tagging and observational data have documented the return of individual females to individual nursery grounds. Adult females of the nurse shark, *G. cirratum*, and the lemon shark, *N. brevirostris*, have been observed repeatedly returning to nursery areas for parturition (Pratt and Carrier 2000, Feldheim *et al.* 2002). Feldheim *et al.* (2004) increased the power to detect philopatric behavior of females by using multi-locus microsatellite profiles of adult females and juvenile lemon sharks to detect sibling groups across years. This approach has the advantage over traditional tagging or observational studies in that once an adult female has been genotyped, philopatric behavior can be detected by catching and genotyping its offspring. In addition, kinship analysis can be used to infer maternally related half siblings across years without ever catching adult females. This is possible in a population where females show strict philopatry to multiple geographically distinct nursery grounds and males do not, because on the nursery ground one would find maternally related kin groups at a much higher frequency than those related paternally.

This latter methodology also allows researchers to detect female reproductive periodicity. Reproductive periodicity is important as it has a direct affect on estimates of lifetime fecundity. If the average litter consists of eight pups, and the ratio of males to females in litters is one to one, it is easy to see that a shark with a 15 year reproductive window might produce 60 female pups in a lifetime. If, however, the reproductive cycle

requires more than a year to complete, a two or three year cycle for example, a female might produce 30 or 20 female pups respectively.

In sandbar sharks, *Carcharhinus plumbeus*, patterns consistent with regional philopatry are described in Chapter 5. In addition, tagging studies have demonstrated that juvenile sharks return to their natal nursery areas every summer for the first 4-12 year of life (Grubbs *et al.* 2007, McCandless *et al.* 2007). However, there has not been an assessment of the fidelity of adult female philopatry to specific nursery grounds. In addition, though a two year reproductive cycle has long been assumed for *C. plumbeus* (Springer 1960, Joung and Chen 1995), there is some evidence that the species may exhibit a non-synchronous periodicity with two years being the minimum female reproductive cycle (Piercy 2007). For these reasons samples of juvenile and adult sandbar sharks were collected from 2003-2006 in three of the most important sandbar shark nursery areas; Delaware Bay (DEL), Chesapeake Bay (CB) and the Eastern Shore lagoons of Virginia (ES) (Grubbs and Musick 2007). Pairwise F statistics were calculated using microsatellite and mtDNA sequence data to look for patterns consistent with female philopatry. In addition, multi-locus microsatellite genotype arrays, from ES and CB, were screened for the presence of kin groups within nurseries across years. Periodicity was assessed by looking for temporal patterns in the detection of these kin groups.

## Materials and Methods:

### Collection, Genotyping and Sequencing

Collections of samples, tissue storage and DNA extraction followed protocols described in previous chapters. A total of 676 juvenile and 40 adult sharks was genotyped at eight microsatellite loci. Amplification and scoring of microsatellite markers are as described in previous chapters. The entire mtDNA control region was amplified and sequenced in a sub-sample of YOY sharks from DEL (N = 52), CB (N = 47) and ES (N = 55) following protocols described in Chapter 5.

Pairwise  $F_{st}$  values based only on haplotype frequency and pairwise  $\Phi_{st}$  using a Tamura Nei model (Tamura and Nei 1993) with gamma a parameter ( $\gamma=0.6524$ ) were calculated from mtDNA sequence data in Arlequin 3.01 (Excoffier *et al.* 2005) with 10,000 permutations at the 0.05 significance level. These values were compared with pairwise  $F_{st}$  values calculated from microsatellite data in Arlequin 3.01. Significance levels were corrected for multiple testing (Rice 1989).

Kin groups were made from 676 juvenile samples taken from CB and ES between 2003-2006 using Colony 1.2 (Wang 2004), a program which uses multi-locus genotype arrays and maximum likelihood methodology to cluster full sibling families within half sibling families. All typing error and allelic dropout rates were set at 0.001. To increase the chance of recovering accurate kin groups, genotypes of 37 adult females caught in ES and 3 adult males caught on the shelf were compared with juveniles. Samples from ES consisted of young of the year (YOY), one and two year old sharks. Only YOY sharks were used in CB to remove the possible confounding factor of juvenile straying. Ages of

juvenile sharks were determined following the protocols outlined in Chapter 3. If there is a two year reproductive periodicity without strict philopatry, then neonates from kin groups should reappear in alternate years regardless of nursery. If female philopatry is strict, these kin groups should only appear in one of the nursery areas.

#### Results:

Pairwise  $F_{st}$  values obtained from microsatellite data were small and non-significant (Table 1). Pairwise  $F_{st}$  and  $\Phi_{st}$  values obtained from mtDNA sequencing data were also small and non-significant (Table 1).

Colony produced an estimate of 165 half sibling groups and 118 full sibling groups with more than one member. Of these, nine groups contained three siblings and 109 contained two siblings. All full sibling groups shared an allele at four or more of the eight loci, while half sibling groups shared an allele at fewer than four loci. Often shared alleles were at the least polymorphic loci, Cli 103 and Cpl 53. For siblings sharing more than four alleles, 33 pairs were born in the same year, 45 were born one year apart, 28 were born two years apart, 20 were born three years apart and seven were born four years apart. There were 24 sibling pairs where one juvenile was caught in CB and one was caught in ES. Three female/offspring pairs were found. Two of the offspring/mother pairs were sampled in the same year. In the other pair, the adult female was sampled in 2007 and her progeny was sampled in 2005. In all three pairs, mother and offspring were sampled in ES. No offspring were detected for the three adult males caught offshore of Virginia.

## Discussion:

The failure of the pairwise comparisons between nursery areas to detect significant mtDNA differentiation could be due to small levels of female straying or an insufficient amount of time for lineage sorting, factors that do not preclude female philopatry. It may be that while most females exhibit strict philopatry, some change nursery grounds either while adult, juvenile or at first reproduction. This type of dynamic would likely lead to an observed homogeneity of haplotype frequencies across nursery grounds even if the level of straying was extremely low. If straying is most common between nursery grounds in the same general area, then comparisons of nuclear and mtDNA data may only be capable of detecting philopatry at large regional scales. Other studies utilizing this methodology to investigate philopatry in sharks have detected regional philopatry (Pardini *et al.* 2001, Keeney *et al.* 2005). While Feldheim *et al.* (2002) were able to demonstrate strict philopatry in some females, the experimental design was not appropriate for examining possible straying that may have affected the other studies.

There is reason to believe, however, that even if female philopatry was strict and there is no straying, that comparisons of mtDNA and nuclear data might still fail. The rate of molecular evolution in the control region of elasmobranchs (0.8%-0.4% per million years Duncan *et al.* 2006, Keeney and Heist 2006) is much slower than in bony fishes (3.6% per million years Donaldson and Wilson 1999) and the nursery grounds in question are very geologically young (~ 10,000 years old, Kraft 1977). In addition, when the number of generations since isolation is less than  $N_{e(t)}$ , daughter populations are likely

to show extensive genealogical polyphyly (Neigel and Avise 1986). If sandbar sharks have a generation time of 20 years and all of the individuals using the current nursery grounds have a common ancestral gene pool, both reasonable assumptions, then there has not been sufficient time for lineage sorting to cause divergence, unless the long term  $N_{ef}$  is less than 500, and there certainly has not been enough time for novel mutations to arise.

The Colony analysis detected sibling and parent-offspring groups in the data. Those groups defined by Colony as half siblings were discounted as noise, because most allele sharing occurred at the least polymorphic loci and shared alleles were usually the ones found in highest frequency in the western North Atlantic population. On the other hand, individuals sharing alleles at 4 or more loci are more likely to be siblings, as they often share less common alleles at more polymorphic loci. It is likely, however, that many of these pairings are the results of sampling error.

Most of the sibling groups detected by colony consist of only two individuals. This is in stark contrast to Feldheim *et al.* (2004) where lemon shark sibling groups featured from 4 - 58 individuals. It is likely that the lack of large sibling groups in the data is caused by the large number of juveniles in the nursery at one time. Feldheim *et al.* (2002), referring to the 897 juvenile sharks sampled over a six year period state, "we systematically and exhaustively sampled young lemon sharks at Bimini." Given that the effective number of breeders using ES in a given year was estimated at 416-798 (chapter 3), and females have 8.4 pups per litter (Sminkey and Musick 1996), an unrealistically large sampling effort would be required to "exhaustively" sample juvenile sandbar sharks

in ES. In addition, if there are on average of 2.3 sires (chapter 2) per litter than the size of full sibling families will be very small.

In lemon sharks, sibling pairs and offspring-parent pairs were found at regular two year intervals (Feldheim *et al.* 2004) whereas intervals in this study were irregular. This does not necessarily mean that female reproductive intervals are irregular. For example, siblings found four years apart may be the result of a female with a two-year cycle whose reproductive effort in the middle was not sampled. However, pairs were found anywhere from one to four years apart, indicating that the assumption of a strict two year reproductive cycle may be incorrect. This inference is consistent with recent work on reproduction in sandbar sharks (Piercy 2007). If reproductive periodicity is irregular in female sandbar sharks, it may be dependent on female condition. This type of dynamic is seen in sea turtles where environmentally mediated changes in prey density may lead to changes in female condition which in turn affects reproductive periodicity (Hays 2000).

Finally, sibling groups were detected across CB and ES. This might suggest that females do not show philopatry to either nursery ground. However, in eight cases, siblings were found in CB and ES during the same year. The groups of juvenile found in CB and ES could be the result of incorrect grouping or could indicate that juveniles themselves move between CB and ES during the year. Movement between the two nursery areas is entirely possible, as sharks could move through the channels and flats of the ES and enter CB without crossing into open coastal waters, where increased predation levels may exist.

In total, the data suggest some level of philopatric behavior, though the scale and strictness of the philopatry are still unclear. The data also suggest that the assumption of a strict two year female reproductive cycle may be incorrect. However, caution must be taken with these conclusions. While some kin groups are likely correct, the number of two individual sibling groups may also indicate that likelihood surfaces were relatively flat, causing the algorithm to group unrelated individuals. A larger suit of microsatellite markers (12 or more) will be needed to generate more robust kin groups. In addition, a larger yearly sampling effort of YOY will be necessary to uncover larger kin groups. Access to more females as they enter nurseries would also help with the assignment of kin groups, as defining offspring parent relationships is easier than defining sibling relationships. Unfortunately, catching females on the nursery grounds is difficult as the impulse to feed is thought to be somewhat repressed in late term pregnant females.

While using kin grouping to infer female reproductive periodicity and philopatry seems promising, the nursery grounds examined in this study pose some methodological challenges. The close proximity of nursery areas makes both female and juvenile straying more likely, a behavior which will obscure patterns of philopatry. In addition, the large number of female's pupping in these nursery grounds means that very large sampling efforts will be required to find kin groups greater than two. Nonetheless, these data do suggest a need to reevaluate reproductive periodicity in this species. This point cannot be stressed enough as the use of a strict two year female reproductive cycle in fisheries models, if incorrect, might overestimate female lifetime fecundity. In turn, the use of these models by managers to implement quotas could further retard the recovery of the stock.

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Table 1 Pairwise F statistics between the lagoons of the Eastern Shore Lagoons of Virginia (ES), Delaware Bay (DEL) and Chesapeake Bay (CB). Estimated F statistic values are below the diagonal, P-values are above the diagonal.

$\Phi_{st}$	ES	DEL	CB
ES	-	0.24936	0.98941
DEL	0.00523	-	0.64815
CB	-0.01641	-0.00655	-
$F_{st}(\text{mtDNA})$	ES	DEL	CB
ES	-	0.51846	0.95446
DEL	-0.00171	-	0.33551
CB	-0.01073	0.00213	-
$F_{st}(\text{micros})$	ES	DEL	CB
ES	-	0.7528	0.10172
DEL	-0.00144	-	0.25997
CB	0.00232	0.00083	-

## Chapter 5

World Phylogeography and Male Mediated Gene Flow in the Sandbar

Shark, *Carcharhinus plumbeus*

**Abstract:**

The sandbar shark, *Carcharhinus plumbeus*, is a large coastal species with a cosmopolitan distribution. Females are thought to show philopatry to nursery grounds while males are not tied down to specific nurseries and may have the potential to migrate long distances, creating a pattern of male mediated gene flow which may lead to discordance in population structures revealed by mtDNA and nuclear markers. While this dynamic has been investigated in elasmobranchs over small scales, it has not been examined at a worldwide scale. Thus we examined patterns of historical phylogeography and contemporary gene flow by genotyping 329 individuals from nine locations throughout this species' range at eight biparentally inherited nuclear microsatellite markers and by sequencing the complete mitochondrial control region. Phylogenetic inference using mtDNA sequencing data results in an Atlantic clade within a paraphyletic Pacific, suggesting dispersal from the Pacific to the Atlantic may have occurred contemporaneously with diversification in the Pacific. Samples from the western Indian Ocean grouped with those from the Atlantic using mtDNA sequencing data and with those from the Pacific using microsatellite data, suggesting that the western Indian Ocean population may have a common origin with the Atlantic and since has experienced male mediated gene flow from the Pacific. Samples taken from Delaware Bay, Chesapeake Bay, the lagoons of the Eastern Shore of Virginia and Gulf of Mexico show no evidence of divergence, supporting the notion that they are part of one large western North Atlantic population. For the remainder of regions, pairwise comparisons using mtDNA sequence data resulted in large significant fixation indices, suggesting female philopatry over long

periods of time. In contrast, pairwise comparisons using microsatellite data resulted in smaller fixation indices, some of which were non-significant. Cumulatively the data suggest that male mediated gene flow has been important in the historical dispersal of the species and continues between some regions in the present.

## Introduction

Molecular phylogeographic study aims to shed light on the historical mechanisms and processes that have led to current distribution of genetic variation within species (Avice 2000) while at the same time providing information about current population structure and gene flow important for management and conservation (Graves 1998). The marine environment provides a particularly challenging arena for such study, as the obvious vicariant boundaries found in terrestrial environments are often lacking, an issue which poses problems both in determining appropriate sampling design as well as analyzing and interpreting results (Waples 1998). In addition, many marine organisms have life histories that allow for long distance dispersal during one or many different life stages (Palumbi *et al.* 1997). Despite this potential for gene flow the use of high resolution molecular markers (sequencing, microsatellites) has demonstrated that there are both cryptic boundaries and fine scale population structure (Barber *et al.* 2000, Reeb *et al.* 2002, Carlsson *et al.* 2004). In species where different male and female reproductive strategies have led to differences in dispersal potential, the situation may be made more complex. To resolve historical and contemporary patterns of biogeography in these species, phylogeographic analysis will need to utilize multiple molecular markers with different modes of inheritance (Karl *et al.* 1992, Palumbi and Baker 1994).

Sharks are a group in which male and female dispersal potential may differ. Many species use nursery areas to increase the survival of their progeny. These areas provide young sharks with a rich array of prey species and more importantly have reduced densities of elasmobranch predators (Springer 1967, Branstetter 1990).

Increased juvenile survival has likely led to the selection for female philopatric behavior. This selection pressure may be particularly high in species which bear live young, as females must balance the increased costs of parental investment with the benefit of increased lifetime reproductive success. Males, on the other hand, have very little parental investment and therefore may be more likely to stray.

Evidence for female philopatry has been reported in multiple shark species. In the lemon shark, *Negaprion brevirostris*, and the nurse shark, *Ginglymostoma cirratum*, females have been observed returning to specific nursery grounds over multiple years (Feldheim *et al.* 2002, Pratt and Carrier 2001). Evidence for philopatric behavior has also been detected in both the white shark, *Carcharodon carcharias*, and the shortfin mako shark, *Isurus oxyrinchus*, where population structure was detected across ocean basins using mtDNA but nuclear microsatellite allele frequency distributions were found to be homogenous (Pardini *et al.* 2001, Schrey and Heist 2003). The most complete picture of differing male and female dispersal comes from a combination of tagging and molecular studies, which demonstrate male mediated gene flow for blacktip sharks, *Carcharhinus limbatus*, in the western North Atlantic, Gulf of Mexico and Caribbean sea (Heuter *et al.* 2004, Keeney *et al.* 2005). Additional studies have demonstrated population structure using mtDNA (Martin 1993, Gardner and Ward 1998, Duncan *et al.* 2006, Keeney and Heist 2006, Castro *et al.* 2007). However, no study to date has examined the implications of female philopatry and male mediated gene flow on historical dispersal processes and current gene flow across a species' entire global distribution.

The sandbar shark, *Carcharhinus plumbeus*, is a large coastal species found in warm temperate and sub-tropical waters and exploited throughout most of its range. It is distributed throughout the Atlantic, Pacific and Indian oceans, though it is noticeably absent from the expanse of Oceania between New Caledonia and the Hawaiian archipelago (Compagno *et al.* 2005). The species has also been reported in the eastern Pacific near the Revillagigedo and the Galapagos Islands but these are likely cases of mistaken identity (J. Musick, personal communication), so the species should be considered absent from the eastern Pacific as well (Fig. 1).

While the distribution of the sandbar shark is considered cosmopolitan, it is also considered discontinuous. However, Springer (1960) suggested that the species may be capable of transoceanic migrations and several lines of evidence support this view. First, previous studies have suggested that animals found in the western North Atlantic and Gulf of Mexico form one panmictic population in which mating takes place off the coast of southern Florida (Springer 1960, Heist *et al.* 1995). Since nursery grounds have been found as far north as Cape Cod, Massachusetts (Castro 1993), some females must migrate a distance of about 1,000 miles to pup. Second, tagged individuals have been recaptured at distances of over 2,000 miles from the site of original capture (Kohler and Turner 2001). This distance is comparable to the shortest distance between North Africa and South America.

There are reasons to expect that if there are migrants between putative populations they might be infrequent and are likely male. The use of nursery areas by females suggests that this species, like many other elasmobranchs, may show female philopatry. Tagging data have already indicated that pups show strong natal philopatry during the

first 3-14 years of life (Grubbs *et al.* 2007, McCandless *et al.* 2007). In addition, differences in characteristics that may have a heritable component have been noted between putative populations. Maximum size and age at maturity varies greatly between sandbar sharks from Hawaii (147 cm PCL, 8 -10 years, Romine *et al.* 2006) and those from the western North Atlantic (172 cm PCL, 15 years, Sminkey and Musick 1995). The mean number of pups per litter also varies between sandbar sharks from Taiwan ( $\mu=7.54$ , Joung and Chen 1995), western Australia ( $\mu=6.5$ , McAuley *et al.* 2007), Hawaii ( $\mu=5.5$ , Daly-Engle *et al.* 2007), the Mediterranean ( $\mu=6.9$  Saiedi, *et al.* 2005) and the western North Atlantic ( $\mu=8.4$ , Sminkey and Musick 1996). Even the rate of genetic polyandry was found to vary between sandbar sharks from Hawaii (40%, Daly-Engle *et al.* 2007) and the western North Atlantic (85%, Portnoy *et al.* 2007). While these differences could be products of environmental influence or sampling error, they may also suggest that these populations are on their own evolutionary trajectories. In species where mating occurs near nursery grounds (Pratt and Carrier 2001), males may remain local to ensure opportunities for copulation. However, in sandbar sharks, mating occurs at offshore locations remote from nursery grounds. In addition, the sexes remain segregated at all other times and males tend to remain offshore (Springer 1960). For these reasons, males may not show any type of site fidelity.

In this study we characterized microsatellite and mtDNA control region variation within and between populations of sandbar sharks worldwide. Since the mtDNA control region is neutral, maternally inherited and haploid, it has a high rate of molecular evolution making it ideal for intraspecific population studies (Brown *et al.* 1979). Microsatellites markers are also neutral and have a fast rate of molecular evolution

making them suitable for intraspecific studies (Jarne and Lagoda 1996), but more importantly, they are biparentally inherited. Discordance between markers in patterns of variation across samples will therefore be very revealing in terms of the affect that sex biased dispersal may have had in shaping the species current distribution. Due to the sandbar sharks' importance as a target species of commercial fisheries throughout most of its range (McAuley *et al.* 2007) and its overexploitation in the western North Atlantic (Musick *et al.* 1993), this study will also be important for designating stock structure on a global basis and examining potential gene flow between stocks. In addition, the sandbar shark has a less tropical distribution than the two other species of carcharhinid sharks for which there has been global phylogeographic work, *Sphyrna lewini* and *Carcharhinus limbatus*. Comparisons made between these studies may therefore help uncover patterns of phylogeography specific to sharks. Finally, comparisons made between this study and other studies involving non-elasmobranch species will be useful in understanding processes that may have affected the distribution of other marine organisms with temperate and subtropical distributions.

#### Materials and Methods:

##### Sample Collection, DNA extraction, Genotyping and Sequencing

Sharks samples in the form of muscle tissue or fin clips were collected from the Pacific Ocean; Hawaii (HI), Taiwan (TW) and Eastern Australia (EAUS), the Indian Ocean; South Africa (SAFR) and Western Australia (WAUS), and the Atlantic Ocean; Delaware Bay (DEL), Chesapeake Bay (CB), Eastern Shore lagoons of VA (ES), and the

Gulf of Mexico (GOM) (Fig 1.). All samples were collected between 2002 and 2006 except for additional samples from GOM which were collected in 1991 and 1993 and all samples from WAUS were collected in 1999. Tissue was stored either in 95% ethanol or 10% DMSO buffer (Seutin *et al.* 1991) at 4° C till extraction. DNA was extracted using a modified Chelex extraction protocol (Estoup *et al.* 1996). After a two minute centrifugation at 16,000g, 0.3ul of the supernatant was used directly as a template for all PCR reactions.

Eight microsatellite markers were amplified for each individual using IR-700 and IRD-800 labeled forward primers. Descriptions of primers and PCR conditions for the six species-specific markers, Cpl53, Cpl90, Cpl128, Cpl132, Cpl166, Cpl169 are reported elsewhere (Portnoy *et al.* 2006, Portnoy *et al.* 2007). Two additional markers, Cli12 and Cli103, originally isolated from the congeneric blacktip shark, *Carcharhinus limbatus*, were run following the protocols outlined in Keeney and Heist (2003). All amplicons were electrophoresed through 25 cm 6.5 % polyacrylamide gels using a LiCor 4200 Global IR<sup>2</sup> system. A 50-350 bp size standard was run in the first, middle, and last lanes of each gel and locus-specific standards were run in every 4th lane. Alleles were scored manually with the aid of Gene ImagIR 4.05 (Scanalytics, Rockville MD). Twenty-five percent of samples were selected and rescored to ensure accurate scoring. Individuals for which more than two loci could not be reproducibly scored were discarded.

The entire mitochondrial control region (1665-1668 bp) was amplified using the primer Pro-L (5'-AGGGRAAGGAGGGTCAAAC-3'), which is complementary to a portion of the proline tRNA located on the light strand, and the primer 282H

(5'AAFGCTAFFACCAAACCT-3') a portion of the 12S rRNA on the heavy strand (Keeney *et al.* 2003). Twenty-five microliter PCR reactions consisted of 20 mM Tris-HCL (pH 8.4), 1.5 mM MgCl<sub>2</sub>, 0.001 mg/μl BSA, 0.2 mM dNTP mix, 25 pmol of each primer, 2ul of template and 0.025 U/ul *Taq* polymerase. Reaction conditions consisted of a denaturation at 95 °C for 4 min followed by 35 cycles at 95 °C for 1 min, 61 °C for 0.35 min and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. PCR products were purified using Qiagen Qiaquick PCR purification kits (Qiagen, Valencia CA). To ensure accurate sequencing of rare haplotypes, two internal primers which when paired with the original primers amplified overlapping fragments, were also developed, CP5'R : (5'-ACCTTAATGAACCAGATGAGCC-3') and CP3'F: (5'-CCTTTAATGGCATATTTATCC-3'). PCR conditions were the same as previously listed except that for Pro-L 5' and CP5'R annealing was at 64.5 °C for 0.45 min and for CP3'F and 282H annealing was at 61.5 °C for 0.35 min.

Purified products were sequenced in the forward and reverse direction using BigDye Terminator v3.1 Cycle sequencing reagents (Applied Biosystems, Warrington UK). Five microliter sequencing reactions consisted of 10-40 ng of template, 0.5ul of BigDye master mix, 1 ul of BigDye 5x Reaction Buffer and 32 pmol of F or R primer. Sequencing conditions consisted of a denaturation at 96 °C for 1 min followed by 25 cycles at 96 °C for 0.1 min, 50 °C for 0.05 min, and 60 °C for 4 min. Amplifications were electrophoresed on an ABI 3130xl sequencer through 70cm capillaries. Results were scored using Sequencing Analysis v 5.2 software (Applied Biosystems, Warrington UK). The resultant SCF curves were imported into Sequencher 3.0 (Gene Codes Corp, Ann Arbor, MI) where consensus sequences of the entire control region were formed by

combining reverse and forward sequences. All consensus sequences were aligned in MacVector 8.1.1 (Accelrys Inc. San Diego CA) using the Clustal W algorithm (Thompson *et al.* 1994).

#### Summary statistics

Conformance to the expectations of Hardy-Weinberg equilibrium was calculated for each microsatellite locus and population in GENEPOP (Raymond and Rousset 1995) using exact tests with 10,000 iterations. Expected and observed numbers of heterozygotes were also calculated in GENEPOP. Number of alleles, allele frequencies and allelic richness were calculated for each locus and putative population with FSTAT (Goudet 2001). Micro-Checker (Oosterhout *et al.* 2004) was used to screen for null alleles and genotyping error.

For control region sequences haplotype diversity ( $h$ ), nucleotide diversity ( $\pi$ ), number of polymorphic sites ( $s$ ), base composition, and the number of transitions, tranversions, insertions and deletions were calculated for each population in Arlequin 3.11 (Excoffier *et al.* 2005).

#### Population Structure and Demographic History

For both microsatellites and control region sequence data, genetic diversity within and among populations and ocean basins was estimated using an analysis of molecular variance (AMOVA) implemented in Arlequin 3.11 with 10,000 permutations (Excoffier *et al.* 1992). Pairwise  $F_{st}$  values were calculated from microsatellite data and pairwise  $\Phi_{st}$  values were calculated from mtDNA sequence data in Arlequin 3.11 with 10,000 permutations at the 0.05 significance level. Significance levels were then corrected for

multiple testing (Rice 1989). To assess for possible population expansion, tests for selective neutrality were run following the methodology of Tajima (1989) and Fu (1997) and conformance of the mismatch distribution to a unimodal distribution was tested (Harpending 1994) in Arlequin 3.11. If Harpending's raggedness ( $H_r$ ) is non-significant and both Tajima's  $D$  ( $D_t$ ) and Fu's  $F$  ( $F_f$ ) are small, negative and significant one can suspect recent rapid population expansion

### Phylogenetic Inference

To better visualize the relationship between populations using microsatellite data, correspondence analysis (Guinand 1996) was implemented in GENETIX v4.05.2 (Belkhir *et al.* 2004). This procedure creates three factors each based on aspects of the allelic composition of the populations. It then plots each individual in relation to these factors.

Alignments of all mtDNA haplotypes were used to create a neighbor-joining (NJ) tree using maximum likelihood distances in PAUP v4.0 (Saitou & Nei 1987, Swofford 2002). The most appropriate model of sequence evolution was selected by Modeltest (Posada 1998). Trees were rooted using the closely related *Carcharhinus falciformis* (Naylor 1992). The robustness of the NJ topology was examined over 1000 bootstrapping replicates using a full heuristic search. Start trees were generated via ten random additions; branch swapping used the nearest neighbor interchange algorithm. Maximum likelihood distances were then used to reconstruct the ML topology in MEGA 4.0 (Tamura *et al.* 2007). Duncan *et al.* (2006) estimated a molecular clock for the control region of 0.8% per million years for the scalloped hammerhead, *Sphyrna lewini*, while

Keeney and Heist (2006) estimated a rate of 0.43% for the blacktip shark, *Carcharhinus limbatus*. Since the former estimate was made for only part of the control region and the later made for the whole control region, estimates of divergence times at major nodes of the tree were made with the molecular clock set at 0.43% sequence divergence per million years.

For comparative purposes distance matrices from microsatellite and mtDNA sequence data were used to form unrooted NJ trees between populations in MEGA 4.0. Cavalli-Sforza chord distance (Cavalli-Sforza and Edwards 1967) and Nei's  $D_a$  (Nei *et al.* 1983) were calculated in MSA analyzer (Dieringer and Schlotterer 2003) from microsatellite data as these measures have been found to outperform other distance measures when used for phylogenetic inference (Takezaki and Nei 1996). Tamura and Nei's (1993) distance method was used to calculate mtDNA sequence divergence between populations in Arlequin 3.11 and corrected net distances were used for NJ trees.

Minimum spanning networks were created in Network software (Fluxus-engineering.com) using the full median joining algorithm (Bandelt *et al.* 1999). Maximum parsimony (MP) analysis was used to remove all unnecessary alternate connections (Polzin and Daneshmand 2003). Support for the most common connections found across trees was calculated by evaluating the percentage of trees in which they appeared.

## Results:

A total of 329 animals from nine localities was genotyped at eight microsatellite loci. After correction for multiple tests only locus Cpl 53 in WAUS deviated significantly from the expectations of Hardy-Weinberg equilibrium ( $p=0.002$ ). This was due to an excess of homozygotes. Micro-Checker confirmed that only this locus showed signs of null alleles. Pairwise comparisons between WAUS and other regions were therefore run with and without Cpl 53. The least polymorphic locus was Cpl 53 which had 10 alleles throughout the entire data set. The most polymorphic locus was Cpl 166, which had 64 alleles throughout the entire data set. Allelic richness averaged across loci was greatest in the SAFR and TW samples, 13.28 and 13.11 respectively. Atlantic samples had smaller average allelic richness than Pacific samples except for HI, which had the smallest average allelic richness (8.45). A table of all Hardy-Weinberg P-values, expected and observed number of heterozygotes and all summary statistics by population and locus are presented in Table 1.

A total of 67 mtDNA haplotypes was found across all samples. The control region varied in size from 1065bp in some Pacific samples to 1068bp in some Atlantic samples. Most of this size heterogeneity was due to indels in long strings of adenine found at the 3' end of the sequence. The sequence was composed of 13.7% guanine, 35.4% thymine, 31.0% adenine and 19.8% cytosine. There were 39 variable sites, eight transversions, 26 transitions and five indels (Table 2). Of 67 haplotypes found, 32 were in the Indo-Pacific region, eight were in the western Indian Ocean and 29 were in the Atlantic Ocean. Of these, two haplotypes were shared between the Indian Ocean and the

Atlantic Ocean. Total nucleon diversity ( $h$ ) was 0.959 and nucleotide diversity ( $\pi$ ) was 0.00475. Haplotype diversity was highest in Atlantic samples and smallest in HI and EAUS. Summary statistics for each population and across populations are presented in Table 3.

AMOVA detected significant population structure for mtDNA sequence data, with a significant component of variance within and between ocean basins ( $\Phi_{SC} = 0.20655$ , %V = 8.98,  $P < 0.00001$  and  $\Phi_{CT} = 0.56544$ , %V = 56.54,  $P = 0.0752$ , Table 4). There was also a significant component of variance found within populations ( $\Phi_{ST} = 0.65520$ , %V = 34.48,  $P < 0.00001$ , Table 4). Pairwise comparisons using mtDNA sequences showed no significant differentiation between collection sites in the Atlantic Ocean. All Atlantic Ocean sites were significantly differentiated from all Pacific and Indian Ocean sites. All Pacific and Indian Ocean sites were significantly differentiated from each other. Pairwise  $\Phi_{st}$  values and P-values can be found in Table 6.

AMOVA detected significant population structure for microsatellite data, with a significant component of variance within and between ocean basins ( $\Phi_{SC} = 0.01073$ , %V = 1.02,  $P < 0.00001$  and  $\Phi_{CT} = 0.04975$ , %V = 44.98,  $P = 0.00489$ , Table 4). A significant component of variance was also found within individual but not within populations (%V = 94.16,  $P < 0.00001$  and %V = -0.15,  $P = 0.5771$ , Table 4). Pairwise comparison using microsatellite data showed no significant genetic differentiation between collection sites in the Atlantic Ocean. All Atlantic Ocean sites were significantly differentiated from all Pacific and Indian Ocean sites. HI showed significant differentiation from all other collection sites. WAUS showed significant differentiation from all other collection sites except TW ( $F_{st} = 0.00107$ ,  $P = 0.31294$ ) before correcting for multiple tests. After

correction, comparisons of WAUS with EAUS and SAFR were also non-significant ( $F_{st} = 0.0476$   $P = .04415$  and  $F_{st} = 0.01006$   $P=0.04435$ ). TW was also not significantly differentiated from SAFR ( $F_{st} = -0.00005$ ,  $P = 0.46995$ ). All pairwise  $F_{st}$  values and their P-values are presented in Table 5.

Correspondence analysis using microsatellite data demonstrated hierarchical population structure. Atlantic Ocean samples clearly group together separate from Pacific and Indian Ocean samples (Fig. 2a). When Pacific and Indian Ocean samples were examined separately HI and SAFR clearly diverge from TW, EAUS and WAUS (Fig. 2b) When HI and SAFR are excluded it is clear that TW, EAUS and WAUS are separate but overlapping (Fig 2c).

#### Demography

$H_r$  could only be calculated for HI, TW, EAUS and SAFR and was not found to be significant for any region. For the remaining regions the algorithm was not able to converge. No samples had a significant  $D_t$ . In fact, only TW had a negative  $D_t$ , a significantly negative  $F_f$  and a non-significant  $H_r$  indicating population expansion. While  $H_r$  could not be calculated for WAUS, the region had a significantly negative  $F_f$  and negative  $D_t$  as did all of the Atlantic samples. EAUS and SAFR both had non-significant  $F_f$  statistics and Hawaii had positive  $D_t$  and  $F_f$ .  $F_f$ ,  $D_t$  and  $H_r$  values are found in Table 7.

#### Phylogenetic Analysis

Likelihood tests suggested a Hasegawa, Kishino and Yano (HKY, Hasegawa *et al.*, 1985) model with six parameters as the most appropriate model of sequence evolution while AIC selected a Tamura Nei (TrN, Tamura and Nei 1993) model with

seven parameters; both included invariable sites ( $I = 0.8576$ ) and a gamma parameter ( $\gamma = 0.652$ ). The latter model was selected as under-parameterization is thought to impose greater bias to tree topologies than over-parameterization (Nylander *et al.* 2004). The NJ tree exhibited a monophyletic Atlantic Ocean, which included all but one of the SAFR haplotypes. The Atlantic clade was nested within a paraphyletic Pacific Ocean, which includes WAUS. Haplotypes from EAUS are sister to the Atlantic clade. HI and EAUS haplotypes appear in two clusters in the Pacific clade. Support values for few nodes were higher than 50%. Estimates of time of divergence at major nodes suggest that the majority of lineage splitting occurred between 600kya and 100kya during the Pleistocene (Fig 3).

Unrooted NJ trees of regions made from Cavalli-Sforza chord distance and Nei's  $D_a$  were highly congruent (Fig 4a and 4b). All of the Atlantic Ocean samples grouped very closely together and are separated from the Indian and Pacific Ocean samples which group together. Both SAFR and HI are closest to TW, EAUS and WAUS which group together. In contrast, the unrooted NJ tree of regions made from mtDNA sequence data show SAFR grouping more closely with Atlantic Ocean samples (Fig 4c).

The minimum spanning network was composed of 44 Steiner trees. There was high support for the majority of connections (Fig 5). Consistent with all NJ analysis the network suggests two haplogroups; Pacific and Atlantic. Three SAFR haplotypes were placed between the larger haplogroups. Another three SAFR haplotypes group within the Atlantic haplogroup and the remaining SAFR haplotype grouped with the Pacific haplogroup. EAUS and HI haplotypes appear in two clusters within the Pacific

haplogroup. Haplotypes for the GOM and the three western North Atlantic collection sites were well mixed.

#### Discussion:

##### Contemporary Population Structure and Gene Flow

This study was able to demonstrate that patterns of population structure on a global scale differ depending on the mode of inheritance of the marker used for inference. While sampling was widespread, covering the species' cosmopolitan range, it was not complete. Within the Atlantic Ocean, all samples came from a limited region in the western North Atlantic. These samples; DEL, CB, ES and GOM, show no signs of divergence using data from either mtDNA sequences or microsatellites. This confirms earlier work using markers with less resolving power, which suggested that the Gulf of Mexico and western North Atlantic were part of one large panmictic unit (Heist *et al.* 1995). This also means that only one Atlantic Ocean population was sampled.

Two haplotypes were shared between the western North Atlantic and the western Indian Ocean. This could be the result of contemporary gene flow around the tip of Africa, a phenomenon seen in other marine species such as the escolar, *Lepidocybium flavobrunnem* (Brendtro *et al.* in press). Alternatively, as many marine species seem to have dispersed around the tip of Africa (Goodbred and Graves 1996, Scoles *et al.* 1998, Bowen 2006) it could reflect a recent shared ancestral gene pool and incomplete lineage sorting. Samples from the eastern Atlantic would be necessary in order to assess which scenario is more likely, since any contemporary gene flow would be between the Indian

Ocean and the eastern Atlantic not the western North Atlantic. In addition, Springer (1960) postulated that there might be gene flow between the eastern and western Atlantic by individuals using equatorial currents. Without samples from the western South Atlantic as well as the eastern Atlantic, such a hypothesis cannot be tested.

Sampling was more complete in the Indo-Pacific and sample numbers per location were fairly large. All pairwise comparisons (aside from those within the Atlantic) show large and significant  $\Phi_{st}$  values, indicating a cessation of contemporary female gene flow. However, a small non-significant pairwise  $F_{st}$  between WAUS and TW suggests that there is contemporary male mediated gene flow.  $F_{st}$  values between EAUS and WAUS were also non-significant, after correction for multiple testing suggesting gene flow.

Since nuclear and mtDNA loci have different modes of inheritance and mtDNA is haploid, one would expect larger pairwise  $F_{st}$  values to be produced using mtDNA (Buonaccorsi *et al.* 2001). In addition  $F_{st}$  values from very polymorphic microsatellites are expected to be small, as  $F_{st}$  values cannot exceed the homozygosity of the markers used to estimate it (Hedrick 1999). However, the pairwise  $F_{st}$  values between WAUS and TW and WAUS and EAUS (0.00107 and 0.00476) are not only non-significant, they are at least an order of magnitude smaller than the corresponding pairwise  $\Phi_{st}$  values (0.17767 and 0.13139). Bias caused by marker type is not likely to explain such a large discrepancy alone; suggesting that contemporary gene flow between these locations is likely creating the observed pattern.

It is important to note that the presence of a null allele was detected at Cpl 53 in the WAUS samples. However, it is not likely that gene flow detected between WAUS

and TW and WAUS and EAUS is an artifact resultant from the null allele. Simulation has demonstrated that when null alleles affect estimates of differentiation, they tend to increase  $F_{st}$  values and estimates of distance (Chapuis and Estoup 2007). To be sure that Cpl 53 had no effect on the results; pairwise comparisons were re-run excluding the locus. The relationship between WAUS and the other regions was unchanged.

Pairwise  $F_{st}$  values were also small and non-significant between SAFR and TW and SAFR and WAUS, suggesting that there may be male mediated gene flow between these regions. A closer look at the results suggests that these values may be due in large part to the relatively small samples size of SAFR (15) which limited the power of the analyses. The pairwise  $F_{st}$  value between WAUS and SAFR is non-significant after correction but is, in fact, larger at 0.01006 ( $P=0.00435$ ) than other  $F_{st}$  values that were found to be significant (EAUS-TW  $F_{st}=0.0073$ ,  $P=0.00218$ ). The notion that these results are caused by sample error is further supported by both correspondence analysis and gene trees made with chord distance. Both show SAFR as distinct and well separated unit from both TW and WAUS. These analyses would therefore be aided by augmenting the SAFR samples and perhaps by sampling the Red Sea to look for gene flow between SAFR and a geographically closer population. Nonetheless it seems that direct contemporary gene flow between SAFR and the Indo-Pacific is highly unlikely.

Together these findings suggest that studies examining population structure in elasmobranchs using only by mtDNA sequencing data may come to the erroneous conclusion that discontinuous populations separated by long geographic distances are completely isolated when, in fact, the observed patterns are the result of female philopatry. These findings are supported by work in the western North Atlantic and Gulf

of Mexico which demonstrated male mediated gene flow and regional female philopatry in blacktip sharks (Keeney *et al.* 2003) as well as work showing philopatry in white sharks and mako sharks (Schrey and Heist 2003, Pardini *et al.* 2001).

#### Historical Dispersal:

Data generated from mtDNA sequencing identified a monophyletic western North Atlantic clade within a paraphyletic Pacific Ocean clade. Using a divergence time of 0.43% per million years (Keeney and Heist 2006), the oldest node in the phylogeny is about 600,000 years old with the colonization of the Atlantic occurring roughly 400 kya. These dates come after both the closing of the Tethys seaway (14 mya) and the rise of Isthmus of Panama (3-4 mya) suggesting that vicariance was not responsible for the species current distribution. This finding is consistent with work done on a number of other discontinuously distributed cosmopolitan marine species such as bluefish and chub mackerel (Graves 1998). Given the placement of the Atlantic haplogroup within the Pacific, it appears that dispersal proceeded from the Indo-Pacific (Briggs 1999). The paraphyly of the Pacific haplogroup, however, is in contrast to previous work with sharks which found deep monophyletic Pacific and Atlantic Ocean lineages (Duncan *et al.* 2006, Keeney and Heist 2006). This suggests that sandbar shark dispersal into the Atlantic Ocean may be more recent and may have occurred on a time scale contemporary with dispersal and diversification in the Pacific Ocean.

It appears likely that dispersal proceeded from the Indo-Pacific into the Atlantic Ocean through the Indian Ocean. This notion is supported by the basal position of several Indian Ocean haplotypes in the Atlantic clade. The alternative pathway from

west to east seems unlikely, especially in light of the species' absence from the eastern Pacific. In addition, mtDNA haplotypes found in HI consistently appear distant from the Atlantic haplogroup, while a clade of EAUS haplotypes is sister to the Atlantic clade. This suggests a common origin of both EAUS and Atlantic Ocean haplotypes in the Indo-Pacific and is more consistent with east to west dispersal through the Indian Ocean, a pattern seen in several other species including sailfish, blue marlin, and bigeye tuna (Graves and McDowell 2003, Martinez *et al.* 2006)

An important finding was that mitochondrial and nuclear inference placed the Indian Ocean samples within different groups. The former places SAFR closest to Atlantic Ocean samples while the latter places SAFR with the Pacific Ocean. This suggests that long after female philopatry had caused western Indian Ocean mtDNA haplotypes to diverge from Indo-Pacific haplotypes; there may have been male mediated gene flow from the Pacific. Alternately, since one SAFR mtDNA haplotype is in the Pacific clade, all western Indian Ocean mtDNA haplotypes may originally have been of Pacific origin and have since been replaced by Atlantic haplotypes. The lack of a reliable mutation rate for microsatellites in elasmobranchs precludes an estimate of whether the mtDNA haplogroups or microsatellite clades are of earlier origin. However, the idea of male mediated gene flow changing the nuclear character of a population is supported by other data in this study. EAUS has the lowest nucleon diversity seen in this study (along with HI) but fairly high allelic richness, a pattern likely created by contemporary male mediated gene flow, from the genetically diverse WAUS, into a population founded by a small number of females. This pattern mirrors that seen in bluefish distributed on both coasts of Australia (Goodbred and Graves 1996). In addition, a multitude of data

supporting female philopatry to nursery grounds in sharks (Heuter *et al.* 2004) suggests that such a dynamic is the more likely explanation for the pattern seen in SAFR.

SAFR is not the only population which shows signs of historical secondary contact via males after a founding event. Both HI and EAUS have divergent mtDNA clades and low haplotype diversity, suggesting that they have been founded by small number of females in several pulses separated in time (Awise 1987). This scenario had been suggested as an explanation for distinct mtDNA lineages in Atlantic blue marlin, Atlantic sailfish, and spotted chub mackerel found in geographically distinct location (Graves 1998). However, while EAUS appears to be receiving contemporary male-mediated gene flow from WAUS, HI has seemingly been isolated for some time, as it appears very divergent from all other sampling locations regardless of marker type. Consistent with this, life history characteristics of the species in HI are divergent from other populations. In HI sandbar sharks mature at smaller sizes, have smaller litters and have different behavior, such as using deep slope area for nurseries instead of embayments and estuaries (Romine *et al.* 2007, Papastamatiou *et al.* 2006)

There was no convincing evidence for recent rapid population growth of sandbar sharks in most of the geographic regions sampled. The long-term stability in the size of these populations is further supported by the fact that neither the minimum spanning network nor the NJ trees exhibit the traditional star shaped phylogeny associated with such population growth (Ball *et al.* 1988). In addition, Atlantic Ocean and Pacific Ocean haplotype divergence appears to have begun at times preceding 400kya. In particular, EAUS, HI and SAFR show no signs of rapid population growth and instead seem to have been populated by multiple dispersal events.

Proposed divergence times must be viewed cautiously as evolutionary rates vary between species (Avice 1994), and thus the rate calculated for *C. limbatus* is likely different than the actual rate for *C. plumbeus*. In addition, support values are weak at many nodes due to the dearth of informative sites found in elasmobranch mtDNA resultant from very slow rates of molecular evolution (Martin 1992). That being said, the molecular clock is not likely to vary greatly and good resolution at some nodes in the haplotypes tree support the conclusion that dispersal occurred during the Pleistocene (1.8mya-11,00kya).

During the Pleistocene there were as many as 20 glacial periods each lasting approximately 100,000 years, followed by much shorter interglacial periods of about 10,000 years (Martinson *et al.* 1987, Dawson 1992). As glacial extent fluctuated, so did the latitudinal extent of tropical and subtropical waters (Savin *et al.* 1975). During the long periods of glaciations, temperature may have restricted the sandbar sharks range, while during the shorter interglacial periods increased temperature at higher latitudes may have allowed pulses of dispersals. This pattern of pulses of range expansion coinciding with periods of glacial lows, followed by isolation during periods of glacial highs has been demonstrated in other fishes (Johnson 2003). However, the data show a pattern of persisting male gene flow after the cessation of female gene flow. Since temperature constraints are likely to be a function of the basic biology of the animal, one would assume that changing temperatures would affect the sexes dispersal potential equally. Perhaps a more important factor was that during glacial periods sea level was as much as 100m lower than today (Shackleton 1987). This would have changed the distribution of inshore habitats, used by most sandbar shark populations as nurseries. During

interglacial periods rising sea level would have flooded many coastal environments creating different inshore nursery areas. As an example, Chesapeake Bay and Delaware Bay, very important contemporary sandbar shark nursery areas, are river beds that flooded after the Younger Dryas (Kraft 1977). An increase in the number of appropriate nursery areas following climate change, especially in the periphery of the species' range, may have allowed some straying by philopatric females and a gradual expansion of the species range. Low levels of straying have been suggested as an explanation for the colonization of new nesting beaches in sea turtles which show strong philopatric behavior (Bowen *et al.* 1992). During the next climate shift these nurseries would drain or flood, affectively halting female dispersal and perhaps dividing formerly continuous distributions. However, if the temperature between these discontinuous groups was appropriate, male mediated gene flow may have continued.

#### Conclusion:

While more regional sampling is needed to uncover the fine detail of sandbar shark population structure and phylogeography, this study demonstrates that different patterns of contemporary gene flow and historical dispersal were observed when markers with different modes of inheritance were used. Historically the species dispersal was likely dependent on female dispersal and this may have been mediated by changes in sea level creating or destroying nursery habitat as well as changing temperatures. This idea seems to be supported by the data, which indicate that there is often a cessation female gene flow prior to a cessation of male gene flow.

A number of prior studies have detected population structure in elasmobranchs using only mtDNA. Since males of many species have great dispersal potential it will be important to reassess their conclusions using nuclear markers especially as they apply to the definition of stock structure.

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Table 1: Summary statistics for eight microsatellite loci within collections from Taiwan (TW), Hawaii (HI), Eastern Australia (EAUS), Western Australia (WAUS), South Africa/Indian Ocean (SAFR), Gulf of Mexico (GOM), Delaware Bay (DEL), Chesapeake Bay (CB) and the lagoons of the Eastern Shore of Virginia (ES). N is number of samples, A is number of alleles, R is allelic richness,  $H_o$  is observed number of heterozygotes,  $H_e$  is expected number of heterozygotes, HW is probability of conformance to the expectations of Hardy-Weinberg equilibrium. Bold values are significant after correction for multiple tests (initial  $\alpha = 0.05$ )

Sample	Locus							
	Cli12	Cli103	Cpl53	Cpl90	Cpl128	Cpl132	Cpl166	Cpl169
<b>TW</b>								
N	48	48	48	48	48	48	48	48
A	11	15	7	22	29	18	45	31
R	7.713	10.392	5.455	15.355	15.371	12.051	21.02	17.499
$H_o$	37	42	28	47	45	44	48	48
$H_e$	35	42	34	45	45	43	47	46
HW	0.7942	0.8733	0.5311	0.6192	0.7393	0.8663	1	0.4152
<b>WAUS</b>								
N	30	30	30	30	30	30	30	30
A	8	15	5	23	16	17	29	25
R	7.203	11.841	4.773	15.899	11.685	12.771	18.884	17.671
$H_o$	22	26	13	30	24	27	28	29
$H_e$	22	27	21	28	27	27	29	29
HW	0.5871	0.4341	<b>0.002</b>	0.1066	0.0416	0.6932	0.3287	0.6961
<b>HI</b>								
N	23	23	23	23	23	23	23	22
A	3	9	5	10	11	8	23	14
R	2.981	8.059	4.461	8.369	8.598	6.799	16.508	12.206
$H_o$	9	20	14	21	15	19	21	21
$H_e$	9	20	15	17	18	18	22	20
HW	0.5689	0.9715	0.9045	0.5278	0.1489	0.7454	0.5945	0.7914
<b>EAUS</b>								
N	43	43	43	43	43	43	43	43
A	22	14	6	24	19	15	42	29
R	7.711	10.623	5.003	14.671	11.93	10.802	21.6	17.992
$H_o$	32	38	29	43	35	37	42	41
$H_e$	28	37	27	40	39	39	42	41
HW	0.9092	0.4366	0.8189	0.3539	0.1129	0.2769	0.8022	0.0693
<b>SAFR</b>								
N	15	15	15	15	14	15	14	15
A	9	13	6	17	12	15	18	19
R	8.798	12.655	5.864	16.389	12	14.524	18	17.998
$H_o$	10	15	10	14	11	13	13	14
$H_e$	12	14	11	14	13	14	13	14
HW	0.1592	0.9778	0.4156	0.7338	0.1224	0.4595	0.147	0.4105

Table 1 cont.

Sample	Locus							
	Cli12	Cli103	Cpl53	Cpl90	Cpl128	Cpl132	Cpl166	Cpl169
<b>GOM</b>	23	23	23	23	23	23	23	23
N								
A	10	7	4	17	10	8	24	23
R	9.195	6.108	3.798	14.308	8.777	7.287	18.443	17.25
H <sub>o</sub>	20	16	13	21	18	20	22	21
H <sub>e</sub>	20	16	14	21	19	18	22	22
HW	0.9452	0.0398	0.6357	0.0931	0.5605	0.9823	0.7528	0.3724
<b>ES</b>								
N	52	52	52	52	52	52	52	52
A	12	8	4	21	15	10	39	34
R	8.413	5.99	3.324	13.263	9.487	7.983	19.979	17.78
H <sub>o</sub>	47	35	29	48	43	41	51	50
H <sub>e</sub>	44	35	30	48	44	43	51	50
HW	0.6298	0.6752	0.5668	0.2592	0.4022	0.5738	0.4097	0.5389
<b>DEL</b>								
N	55	55	55	55	55	55	55	55
A	10	10	4	20	13	13	47	33
R	7.778	6.315	2.701	12.122	9.79	8.891	20.82	17.096
H <sub>o</sub>	46	40	26	49	47	55	77	51
H <sub>e</sub>	48	35	29	49	47	54	75	52
HW	0.0941	0.365	0.7084	0.7667	0.998	0.183	0.9909	0.1878
<b>CB</b>								
N	47	46	46	47	47	47	47	47
A	10	7	3	20	14	10	36	31
R	7.949	5.413	2.773	12.158	9.448	7.855	20.064	16.847
H <sub>o</sub>	43	23	23	46	41	41	44	45
H <sub>e</sub>	41	22	25	42	41	39	46	45
HW	0.953	0.5597	0.7576	0.5382	0.659	0.5617	0.3896	0.5713

Table 2: Polymorphic nucleotide positions for 67 sandbar shark haplotypes. Only 39 variable sites are displayed, deletions are indicated with (-).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Hap	6	8	9	9	6	7	8	3	8	2	1	0	1	6	1	1	2	1	8	8	5	3	9	4	7	6	0	1	2	2	3	5	7	5	8	6	3	7	5
PA	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PB	C	T	T	T	G	C	T	G	T	G	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	G	A	G
PC	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	A	A	G
PD	C	T	T	T	G	C	T	G	T	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	G	A	G
PE	C	T	T	T	G	C	T	G	T	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PF	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	G	T	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	A	A	G
PG	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	G	A	G
PH	C	T	T	T	G	C	T	G	T	A	A	T	C	C	C	G	T	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	A	A	G
PI	C	T	T	T	G	C	T	G	T	A	A	T	C	T	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	A	A	G
PJ	C	T	T	T	G	C	T	G	T	A	A	T	C	T	C	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PK	C	T	T	T	G	C	T	G	C	A	A	T	C	T	T	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PL	C	T	T	T	G	C	T	G	T	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	A	A	G
PM	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	G	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PN	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PO	C	T	T	T	G	C	T	G	T	A	A	T	C	T	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	A	G	C	C	-	A	A	A	G
PP	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	G	T	G	T	G	G	G	T	C	A	A	A	-	T	A	A	G	C	C	-	A	A	A	G
PQ	C	T	T	T	G	C	T	G	C	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	T	A	-	T	A	A	G	C	C	-	A	A	A	G
PR	C	T	T	C	G	C	T	T	A	A	T	C	C	C	A	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G	
PS	C	T	T	T	G	C	T	G	T	A	A	T	C	T	C	A	C	G	T	G	G	G	T	C	A	A	-	-	T	A	-	G	C	C	-	A	A	A	G
PT	C	T	T	T	G	C	T	G	T	A	A	T	C	C	C	A	C	G	A	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	A	A	G
PU	C	T	T	T	G	C	T	G	T	A	A	T	C	T	C	A	C	G	T	G	G	G	T	C	A	A	A	-	T	A	-	G	C	C	-	A	G	A	G
PV	C	T	T	T	G	C	T	G	T	A	A	T	C	T	C	G	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	A	A	G
PW	C	T	T	T	G	C	T	G	T	A	A	T	C	C	C	G	C	G	T	G	G	G	T	C	A	A	A	A	T	A	-	G	C	C	-	A	G	A	G





Table 3: Summary statistics for mtDNA haplotypes by population and across all populations (Total). N is number of samples, H is number of haplotypes, s is number of variable sites, h is nucleon diversity and  $\pi$  is nucleotide diversity

	N	H	s	h	$\pi$
TW	46	16	9	0.8995	0.002105
WAUS	25	13	10	0.9300	0.002237
HI	23	4	4	0.5415	0.001609
EAUS	43	10	12	0.5415	0.002225
SAFR	15	8	17	0.8667	0.004677
GOM	23	13	8	0.9526	0.002067
ES	52	16	12	0.9080	0.001962
DEL	55	18	11	0.9091	0.002098
CB	47	22	14	0.9315	0.002192
Total	329	67	39	0.9590	0.004750

Table 4: Results of hierarchical AMOVA using mtDNA sequencing and microsatellite data. DF is degrees of freedom, SSD is the sum of squares, VC is the variance component, %V is percent of variance.

<b>Comparison</b>		<b>DF</b>	<b>SSD</b>	<b>VC</b>	<b>%V</b>	<b><math>\Phi_{st}</math></b>	<b>P-Value</b>
<b>mtDNA</b>							
Among Ocean Basins	FCT	1	289.972	1.73671	56.54	0.56544	0.00752
Among Populations within Oceans	FSC	7	75.334	0.27569	8.98	0.20655	< 0.00001
Within Populations	FST	320	338.89	1.05903	34.48	0.65520	< 0.00001
<b>Microsatellite</b>							
Among Ocean Basins	FCT	1	65.416	0.17629	44.98	0.04975	0.00489
Among Populations within Oceans	FSC	7	41.467	0.03614	1.02	0.01073	< 0.00001
Among Individuals Within Populations	FIS	327	1087.42	-0.00543	-0.15	-0.00163	0.57710
Within Individuals	FIT	336	1121.0	3.33631	94.16	0.05842	< 0.00001

Table 5: Pairwise  $F_{st}$  values for microsatellite data. Below diagonal are  $F_{st}$  values, above diagonal are P-values. Italic  $F_{st}$  are significant at  $\alpha=0.05$ , bold  $F_{st}$  are significant after sequential Bonferroni correction.

	TW	WAUS	HI	EAUS	SAF	GOM	ES	DEL	CB
TW	-	0.31294	0.00000	0.00218	0.46995	0.00000	0.00000	0.00000	0.00000
WAUS	0.00107	-	0.00000	0.04415	0.04435	0.00000	0.00000	0.00000	0.00000
HI	<b>0.04214</b>	<b>0.05400</b>	-	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
EAUS	<b>0.00703</b>	<i>0.00476</i>	<b>0.06242</b>	-	0.00069	0.00000	0.00000	0.00000	0.00000
SAF	-0.00005	<i>0.01006</i>	<b>0.05618</b>	<b>0.01545</b>	-	0.00000	0.00000	0.00000	0.00000
GOM	<b>0.04282</b>	<b>0.04633</b>	<b>0.10496</b>	<b>0.04767</b>	<b>0.03775</b>	-	0.66251	0.46164	0.10900
ES	<b>0.04379</b>	<b>0.04827</b>	<b>0.09748</b>	<b>0.05508</b>	<b>0.03486</b>	-0.01520	-	0.75280	0.10712
DEL	<b>0.04945</b>	<b>0.05386</b>	<b>0.10758</b>	<b>0.05878</b>	<b>0.04197</b>	-0.00033	-0.00144	-	0.25997
CB	<b>0.05914</b>	<b>0.06317</b>	<b>0.11887</b>	<b>0.06662</b>	<b>0.05200</b>	0.00385	0.00232	0.00083	-

Table 6: Pairwise  $\Phi_{st}$  values for mtDNA control region sequence data. Below diagonal are  $\Phi_{st}$  values, above diagonal are P-values.

Bold  $\Phi_{st}$  are significant at  $\alpha=0.05$  after sequential Bonferroni correction.

	ES	DEL	CB	GOM	WAUS	EAUS	TW	HI	SAF
ES	-	0.24938	0.98941	0.76339	0.00000	0.00000	0.00000	0.00000	0.00000
DEL	0.00523	-	0.64815	0.15404	0.00000	0.00000	0.00000	0.00000	0.00000
CB	-0.01641	-0.00655	-	0.6039	0.00000	0.00000	0.00000	0.00000	0.00000
GOM	-0.01477	0.01809	-0.00916	-	0.00000	0.00000	0.00000	0.00000	0.00000
WAUS	<b>0.66646</b>	<b>0.65216</b>	<b>0.64901</b>	<b>0.67455</b>	-	0.00000	0.00000	0.00000	0.00000
EAUS	<b>0.67610</b>	<b>0.65709</b>	<b>0.66041</b>	<b>0.67812</b>	<b>0.13139</b>	-	0.00000	0.00000	0.00000
TW	<b>0.67663</b>	<b>0.66051</b>	<b>0.66226</b>	<b>0.69056</b>	<b>0.17767</b>	<b>0.28807</b>	-	0.00000	0.00000
HI	<b>0.67429</b>	<b>0.65929</b>	<b>0.65642</b>	<b>0.69129</b>	<b>0.41038</b>	<b>0.46710</b>	<b>0.31442</b>	-	0.00000
SAF	<b>0.46851</b>	<b>0.42839</b>	<b>0.43792</b>	<b>0.44121</b>	<b>0.61651</b>	<b>0.58801</b>	<b>0.65024</b>	<b>0.66568</b>	-

Table 7: Measures of possible population expansion by region. Tajima's D is  $D_t$ , Fu's F is  $F_f$ , and Harpending's raggedness is  $H_r$ . Significant values at  $\alpha=0.05$  are bolded.

	$D_t$	$F_f$	$H_r$
TW	-0.03179	<b>-7.86533</b>	0.0380
WAUS	-0.46787	<b>-6.70031</b>	NA
HI	1.71525	1.54470	0.2670
EAUS	-0.48984	-1.54305	0.1220
SAFR	-1.51510	-0.18496	0.0611
GOM	0.05686	<b>-7.82349</b>	NA
ES	-0.45048	<b>-7.84839</b>	NA
DEL	-0.19745	<b>-9.64429</b>	NA
CB	-0.66653	<b>-17.0725</b>	NA

Figure 1: Map of worldwide sampling locations. Sampling effort is bolded numbers, distribution of species is red shadow, map adapted from Compagno *et al.* (2005).

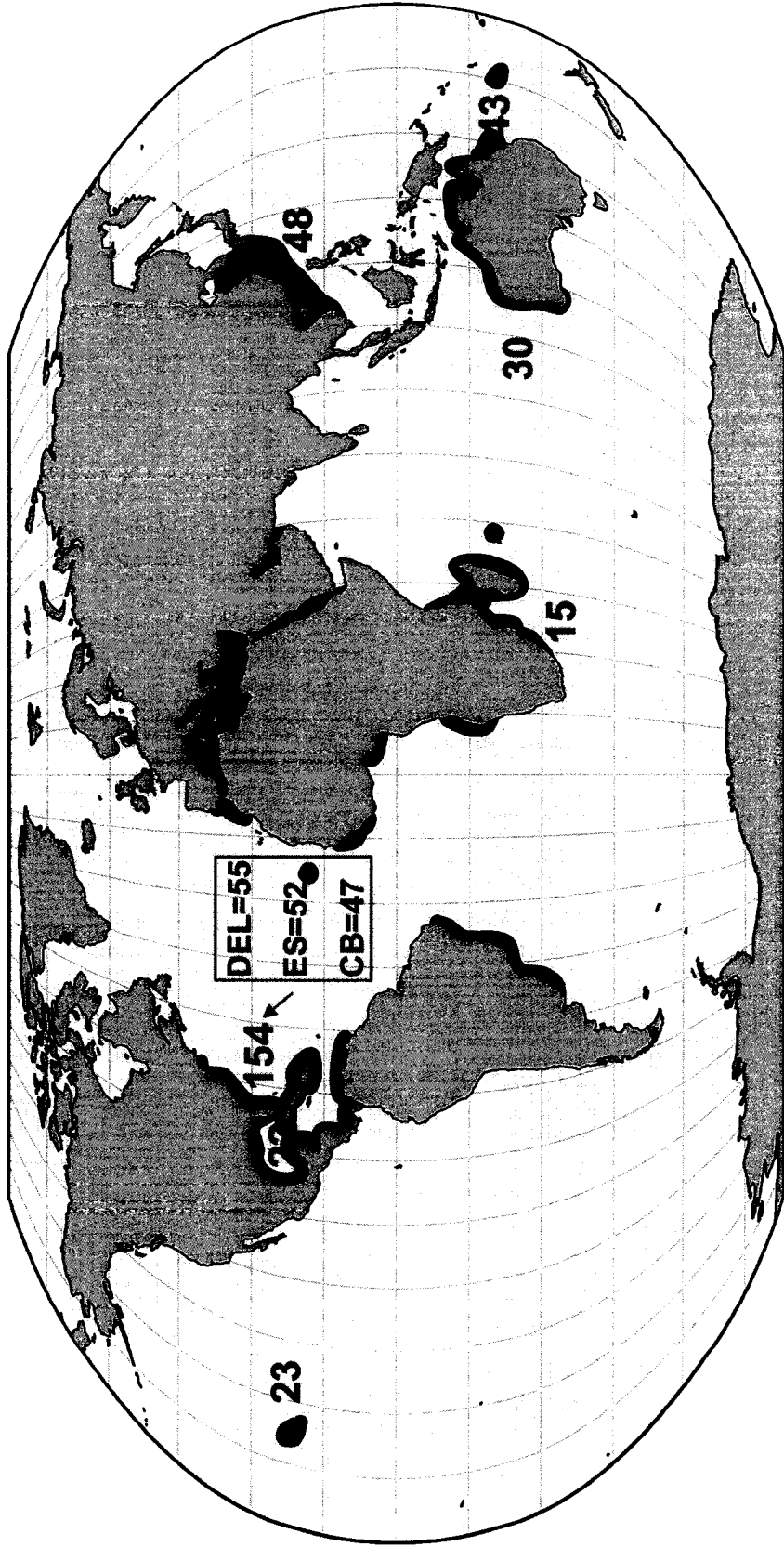


Figure 2: Correspondence analysis of populations using microsatellite data; 2a) All regions, 2b) Atlantic Ocean samples excluded, 2c) Only TW, WAUS and EAUS. Yellow is TW, blue is WAUS, white is EAUS, grey is HI, pink is SAFR, light green is CB, Brown is DEL, Black is ES, and dark green is GOM.

- TW
- WAUS
- EAUS
- HI
- SAFR
- CB
- DEL
- ES
- GOM

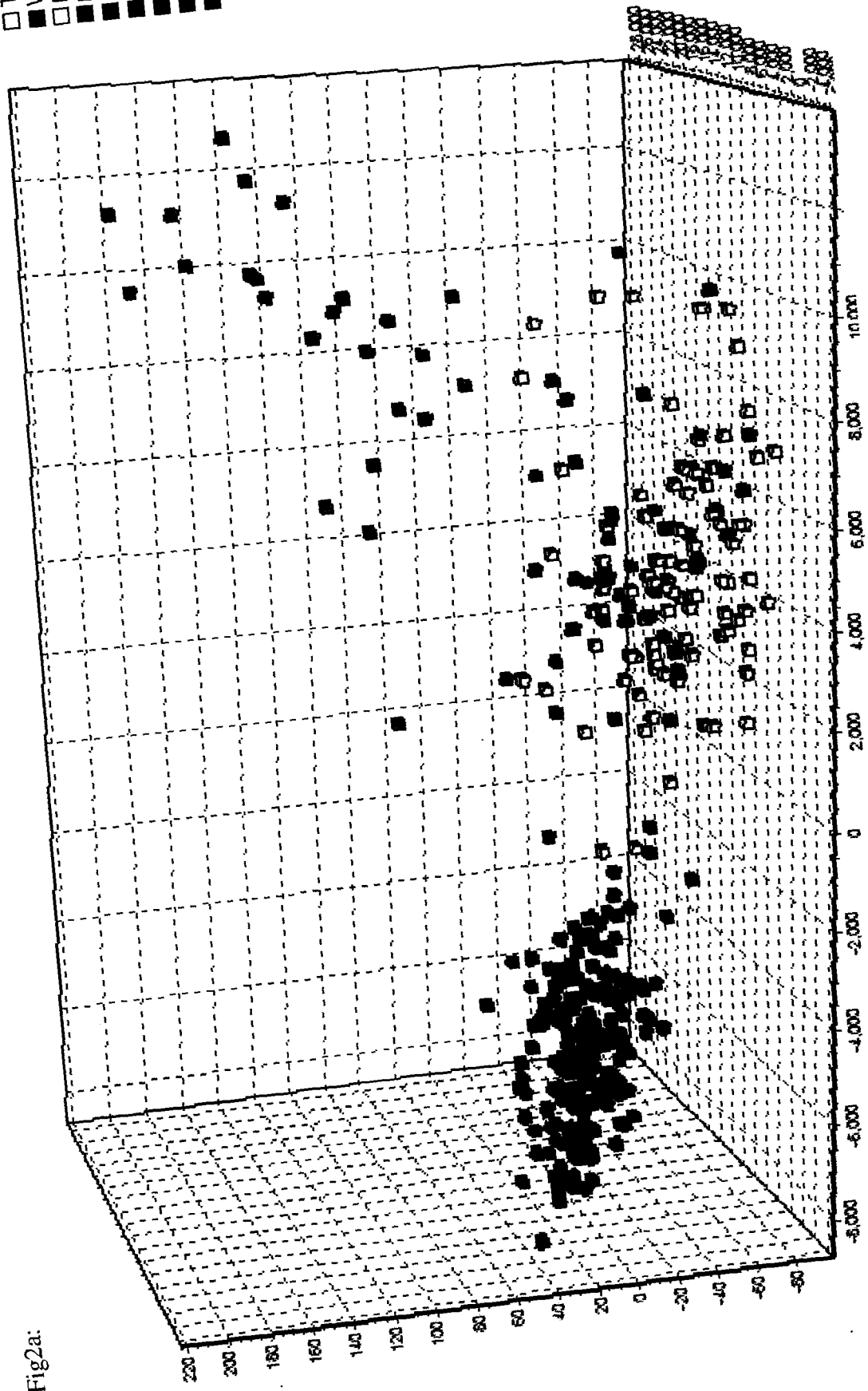


Fig2a:

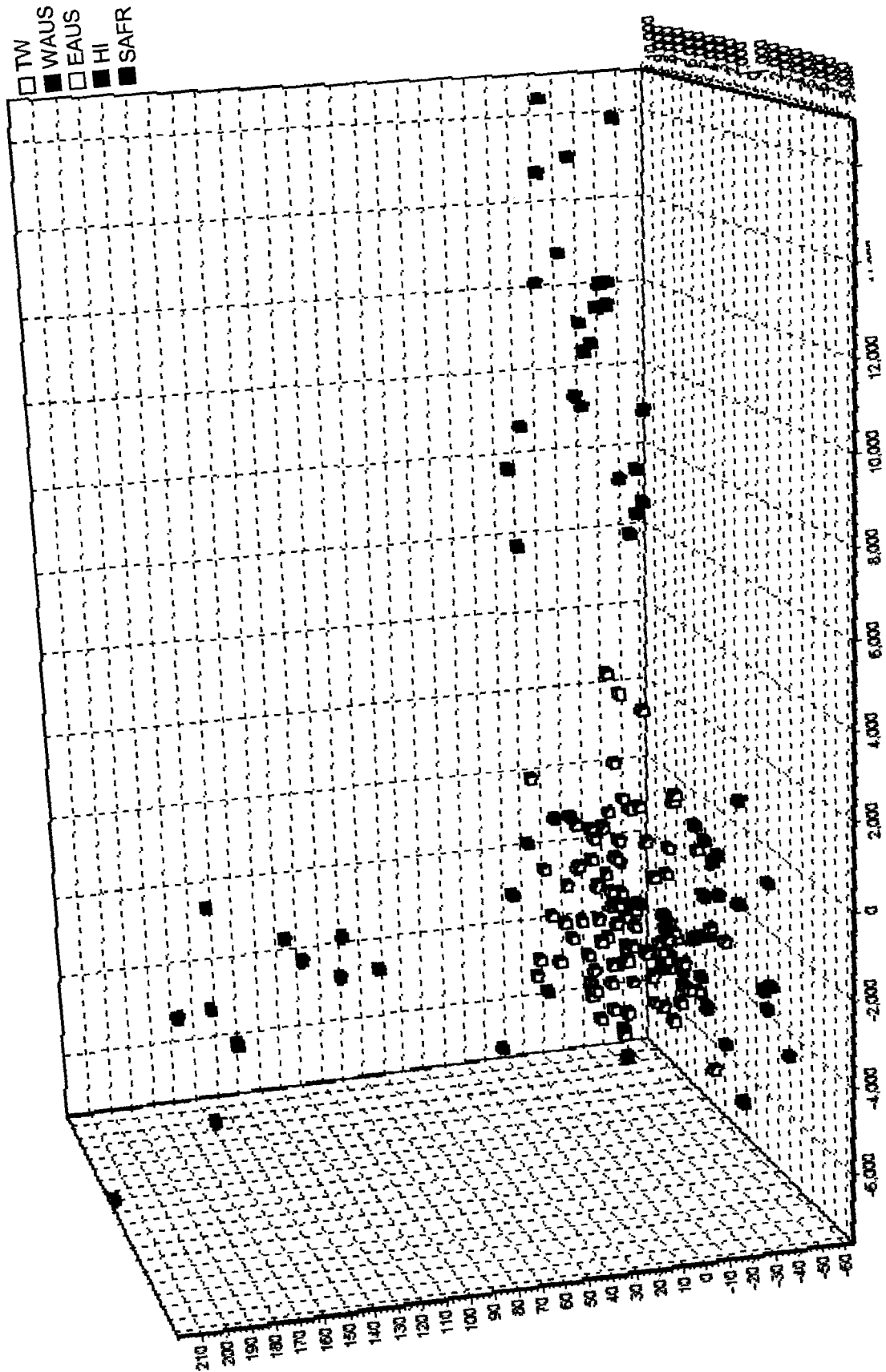


Fig 2b:

□ TW  
■ WAUS  
□ EAUS

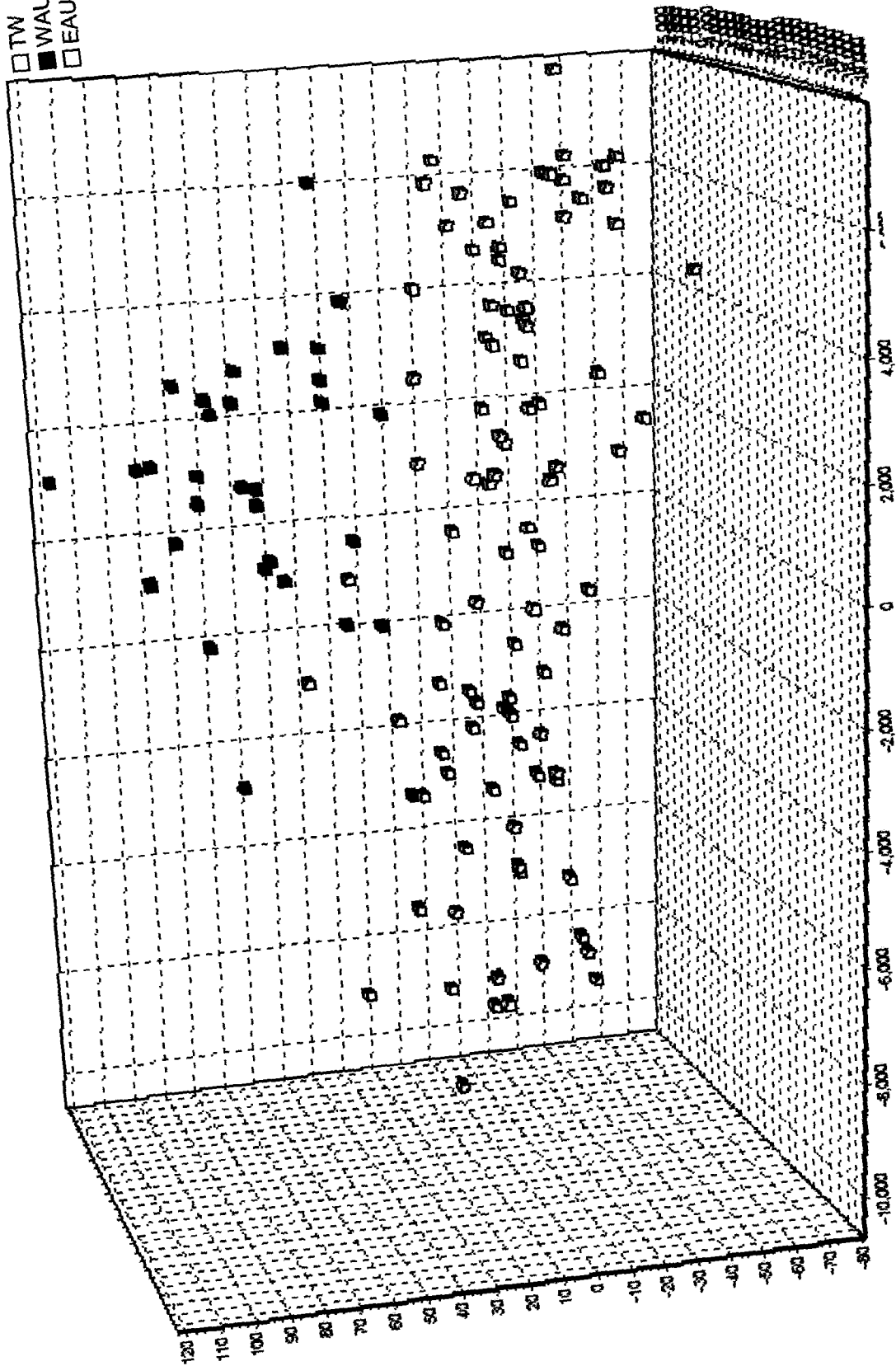


Fig 2c:

Figure 3: Neighbor-joining tree of all haplotypes (67) found in study. Support values > 30% (*italics*), generated from 1,000 bootstrap replicates, are displayed above branches. Estimated divergence times (**bolded**) of several nodes made using divergence estimate of 0.43% per million years (Keeney and Heist 2006).

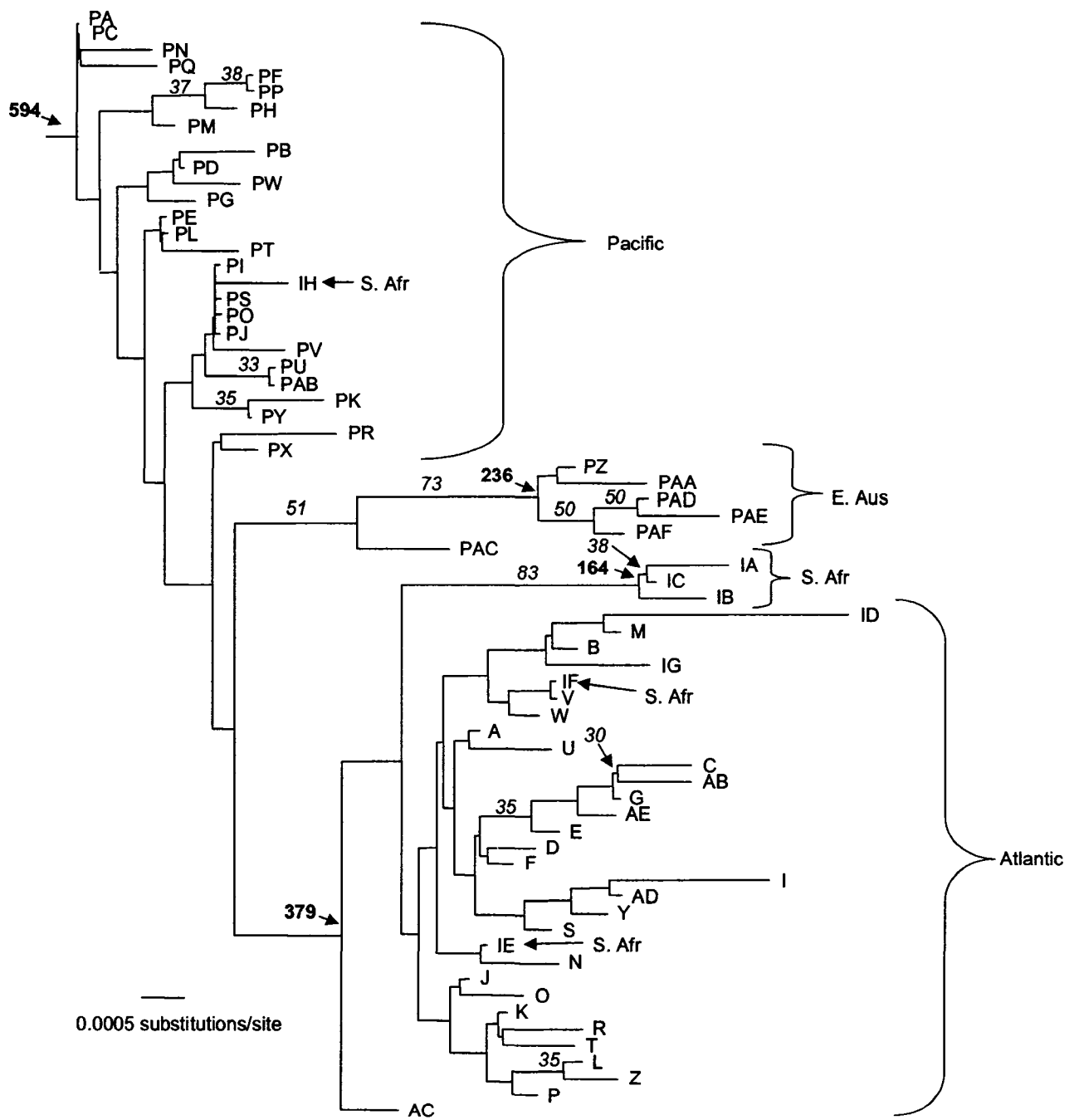
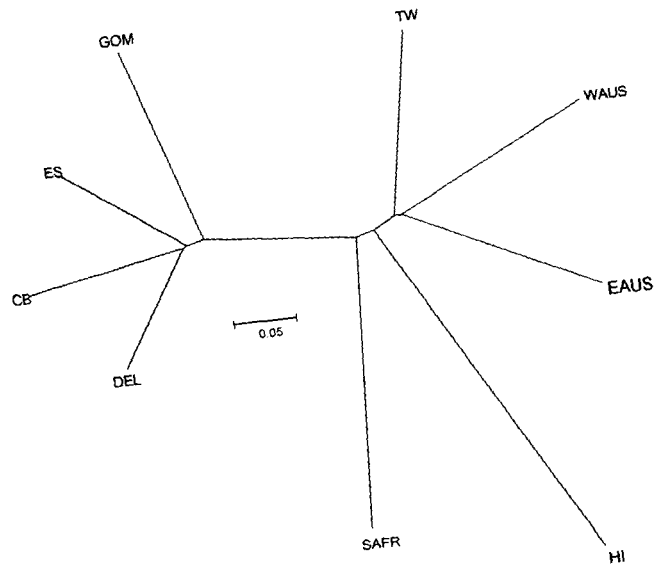
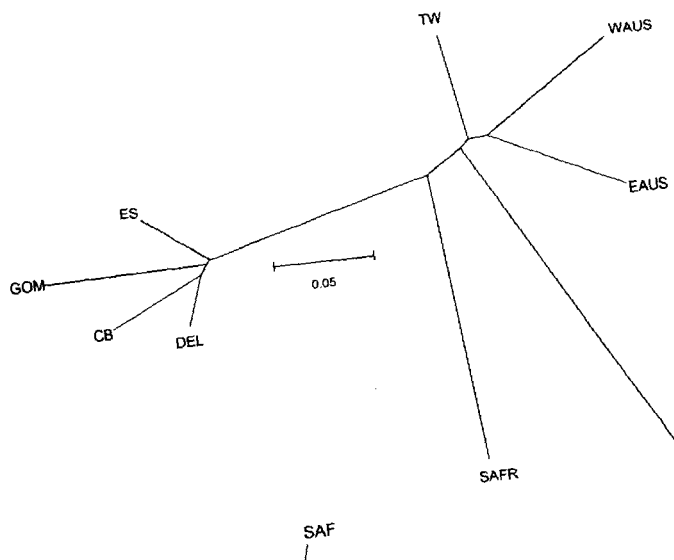


Figure 4: Unrooted neighbor-joining trees for worldwide samples using microsatellite and mtDNA sequencing data. 4a: Cavalli-Sforza chord distance. 4b: Nei's  $D_a$ . 4c: corrected distance using TrN model of sequence evolution.

4a:



4b:



4c:

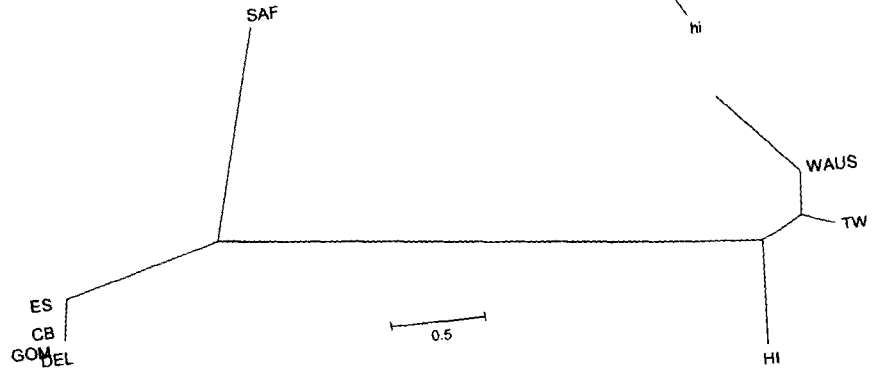


Figure 5: Minimum spanning network of 67 haplotypes found in this study created using the median joining algorithm. Support values (percentage of Steiner trees with connection) listed to the right or above connections. Connections in torso are green, connections exterior to torso are red.



## CONCLUSIONS

The purpose of this study was to generate information about reproduction and behavior in the sandbar shark that would be useful for conservation and management. Using a molecular approach I was able to generate data that would have been difficult to acquire with conventional observational studies. In addition, the molecular markers used in this study, microsatellites and control region sequences, allowed me to ask questions at differing levels of resolution, from fine scale investigations that dealt with individual reproductive success to broad investigations that looked at historical processes affecting the sandbar shark's contemporary distribution. In so doing, this dissertation not only provides important biological information about the sandbar shark but also demonstrates the power and utility of molecular techniques to provide a wide variety of information which complements data acquired from more standard techniques used in fisheries science. The major findings of this research and suggestions for future research are presented below.

### I. Polyandry

Although litter sizes in *Carcharhinus plumbeus* were smaller than those previously reported for other elasmobranchs (Saville *et al.* 2002, Chapman *et al.*, Feldhiem *et al.* 2001), multiple sires were found in 17 of the 20 litters examined. Even though polyandry appears to be the dominant reproductive mode in sandbar sharks in the western North Atlantic, no direct benefit to females seems to explain the pattern. This leaves indirect benefits or convenience polyandry as the most likely explanation for female remating. Since intraspecific competition for mates is intense and breeding is coercive in nature (Pratt and Carrier 2001), female mate choice may be limited and

female remating may be a form of convenience polyandry (Thornhill and Alcock 1983). This also may suggest that long standing conflicts over mating rate have led to adaptations, such as sperm storage, which allow females some form of cryptic mate choice. However, ruling out indirect benefits to polyandry entirely is neither warranted nor appropriate.

In general, testing hypotheses about the benefits and costs associated with elasmobranch mating systems is difficult. The size and highly vagile nature of many species, along with long generation times, prevent typical laboratory manipulations that might allow one to test hypotheses about indirect benefits. This leaves field based inquiries as the only type of investigation available to elucidate the reasons for female remating. One type of field based methodology requires measuring the survival rate and subsequent reproductive success of progeny from singly and multiply mated litters from known females to make an appraisal of fitness. This has recently been attempted with the lemon shark, *Negaprion brevirostris* (DiBatissta *et al.* 2008), but the survival rate was only examined over the first several years of life. No benefits were detected, likely due to the difficulty of relating measures of juvenile survival to the fitness of their mother in a meaningful way. Given that species like sandbar sharks take about 15 years to reach maturity, correctly measuring increased fitness due to indirect benefits would be extremely difficult and require considerable time.

A more fruitful line of research concerning the benefits and costs associated with elasmobranch mating system will come from comparative studies that look at multiple species in different environments to find consistent demographic and environmental variables associated with intraspecific differences in female remating rate. A study

conducted simultaneously to this one in Hawaii saw lower rates of polyandry than this study, with the majority of females engaging in genetic monandry (Daly-Engle *et al.* 2007). While it may be tempting to compare these studies and argue that differences in operational sex ratio and density of breeders could explain the discrepancy, it is important to note that litter sizes were on average smaller in Hawaii and the markers used by the investigators had less resolving power. This means that the true rate of polyandry may have been underestimated. Nonetheless, these types of comparison are likely the most productive avenue of study and more should be conducted.

The importance of understanding mating rate goes beyond evolutionary biology. The effect that exploitation will have on mating rate will be determined in large part by which sex is dictating the rate. For example, if coercive male mating tactics causes remating in females, and the success of males is correlated with the density of breeders on the mating ground, then exploitation could lower female remating rate by lowering the density of breeders. If such a dynamic leads to the exclusion of smaller, less experienced males, there may be a decrease in the effective number of breeders. Over time this could result in a decrease in effective size (Martinez *et al.* 2000). On the other hand, if remating rate is dictated by females and is not density dependent, exploitation may have little impact on the effective size.

## II. Effective size

The ratio of effective size ( $N_e$ ) and effective number of breeders ( $N_b$ ) to census size ( $N_c$ ) in the sandbar shark is close to 0.5, following the expectations of organisms with overlapping generations (Nunney 1993). This suggests that there is fairly even

reproductive success within and between the sexes. In addition, this ratio suggests that exploitation is likely to remove genetic variation while removing biomass, a phenomenon that is less likely to occur in bony fishes, where  $N_e/N_b$  is several orders of magnitude smaller (Hedrick 2005). These results also suggest that it may be useful for fisheries managers to monitor  $N_e$  using genetic techniques as a proxy for abundance.

This investigation was confined to studying  $N_b$  at two nursery grounds and  $N_e$  as it applied to those breeders over short periods of time. To better understand the relationship between  $N_e$  and  $N_c$  over longer time scales it will be necessary to employ methodologies, like the Jorde and Ryman (1995) temporal method, across many consecutive cohorts; likely ten or more. In addition, by comparing yearly  $N_b$  to  $N_c$  over these time periods, the appropriateness of using of this technique to monitor biomass can be better assessed.

### III. Periodicity and Philopatry

I was unable to detect differentiation between nursery grounds that would have been indicative of strict female philopatry, but kin groups may have been observed within and across nursery areas. Though the methodology was imperfect, due to the lack of larger sample sizes and more polymorphic microsatellite markers, some of these small kin groups observed across years were likely correct. The data suggest that reproductive periodicity in the sandbar shark may be irregular, an idea that is supported by non-molecular data (Piercy 2007). While it seems unlikely that females are capable of reproducing in consecutive years, it does not seem unreasonable that females require a one or two year quiescent period and that the length of the quiescent period is dependent

on a female's ability to acquire the necessary energy resources to develop a litter. In addition, it appears that CB and ES may not be separate nursery grounds, or that females do not show philopatry to one or the other.

The work done in this dissertation must be interpreted cautiously, as there are many confounding factors that may have contribute to the observed pattern. However, it does highlight the need for more work in this area. The rate of female straying between nursery grounds remains an important parameter that has still not been defined for any elasmobranch. If female sandbar sharks do have an irregular reproductive periodicity, future work should focus on describing the percentages of females having one, two, or three or greater year cycles. In addition, it will be important to understand the factors that lead to changes in periodicity; possibilities include food availability, age, and individual difference in periodicity due to some sort of heritable component.

#### IV. Phylogeography

This study adds valuable information that can be compared to previous phylogeographic studies of sharks (Duncan *et al.* 2006, Keeney and Heist 2006, Castro *et al.* 2007) that relied solely on mtDNA sequence data. In this case, the use of markers with different modes of inheritance, mtDNA and microsatellites, has revealed the importance of regional female philopatry and male mediated gene flow on both historical and contemporary time scales. On a historical time scale it appears that surges of female dispersal may have been important in establishing new populations, and that periods of secondary contact may allow divergent mtDNA lineages to occupy geographically distinct areas. Male mediated gene flow appears to have the capacity to continue long

after female movement has been stopped; a conclusion corroborated by contemporary nuclear gene flow between locations that appear isolated when mtDNA sequencing data is used.

Using maternally and biparentally inherited markers is advisable when investigating population structure in elasmobranchs. For management purpose defining stock structure using only mtDNA sequencing is likely to lead to faulty conclusions regarding evolutionary potential and population size. At the same time, studies using only microsatellite data will not detect regional philopatry and may overestimate the resiliency of a population to the harvesting of females. In addition, studies using only microsatellite data may underestimate the impact of habitat degradation to species like the sandbar shark, where the presence of appropriate nursery grounds may be so important that it has determined the species modern distribution.

#### Future directions

This dissertation has generated important information about the sandbar shark for conservation and management purposes, and it has brought many other questions into focus. To address these questions future researchers will need to employ many of the strategies used in this research. Phylogeographic studies need not only to employ the two marker approach but should attempt to sample juveniles from nursery areas, when known, as well as adults. This type of methodology will be difficult, as acquiring samples from much of the world requires a considerable effort. In many regions the expertise required for such sampling is absent, but if this type of sampling on a global scale is accomplished will result in a very complete picture of gene flow. In coordinating

such work researchers could begin to accumulate data about  $N_e$  and polyandry across populations in an attempt to find demographic and environmental variables that may explain observed differences. In addition, researchers should begin to examine differences in heritable characters between populations. This will be difficult for many species. However, elasmobranch husbandry has improved greatly and raising animals from divergent populations in identical conditions is an exciting possibility, especially for smaller species. Research that examines things like patterns of gene flow or polyandry across species and across regions will also help to elucidate aspects of species biology that shape reproductive behavior.

A common thread in all of these projects should be the integration of molecular and non molecular techniques. As this dissertation has clearly demonstrated, molecular techniques have great utility and versatility for answering questions of conservation and management concern. It is clear, however, that these techniques are most powerful when used as part of an integrated approach and using them in this context will be important for the continued exploration of elasmobranch biology and conservation.

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