POPULATION STRUCTURE, GENE FLOW, AND HISTORICAL DEMOGRAPHY OF A LARGE COASTAL SHARK, THE BULL SHARK (*CARCHARHINUS LEUCAS*) IN THE NORTHWESTERN ATLANTIC

A Thesis

by

ERICA LANE ATKINS

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Chair of Committee,	Jaime R. Alvarado-Bremer
Committee Members,	R.J. David Wells
	Jessica Labonté
Head of Department,	Anja Schulze

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ABSTRACT

Bull Shark, *Carcharhinus leucas*, reproductive patterns are believed to be characterized by female philopatry towards their natal site, while males migrate longer distances. This behavior linked to sex-biased dispersal can result in differing patterns of population structure between markers that differ in the mode of inheritance. This study tested the hypothesis that Bull Shark exhibit male-biased dispersal by characterizing and comparing the maternally inherited mitochondrial DNA Control Region (mtDNA CR) to single nucleotide polymorphism (SNP) data, from the biparentally inherited nuclear DNA genome. Population structure and historical demography were assessed using representative samples throughout the entire Gulf of Mexico (GoM) and Atlantic coastline of the US (NWA), with reference sequences from the Caribbean, eastern Pacific, and western Pacific.

Here we report significant population differentiation within the mtDNA CR between the Gulf of Mexico (GoM) and Atlantic coast (NWA) of the US (F_{CT}, $p < 0.05$), though no structuring was observed within either region. The Florida Keys was identified as containing significantly higher levels of haplotype diversity than both the GoM and NWA, indicating it may act as a mixing zone between the two regions. Interestingly, the Caribbean samples from the San Juan River in Nicaragua diverged as a distinct clade, suggesting Nicaragua may have a genetically unique freshwater population, or may be mixing with the southern Atlantic populations.

Historical demography estimates were calculated for both the GoM and NWA. Both Tajima's D and R2 neutrality statistics suggest a constant population size, and lack support for recent expansion. The estimate of female effective population size was much lower in the GoM (105k) than the NWA (194.5k), though now surprising given the extremely low levels of haplotype and nucleotide diversity observed within the GoM.

STRUCTURE analyses of SNP data using 18,174 variant sites identified discrete populations between the southern GoM (Campeche, Mexico) and the rest of the GoM. Surprisingly, 50% of the samples from North Carolina were assigned to the same cluster at the southern GoM samples. Given the discrepancy in observed population structuring within the GoM between mtDNA and SNP data, we concluded the observed differences in gene flow between the southern GoM and NWA were the result of male-biased dispersal. The structuring of male dispersal between the southern GoM and NWA (North Carolina) has not yet been described in other shark species. The distinct subdivision of each sex creates uniquely restricted gene flow that should be considered and implemented in future conservation efforts to best maintain viability of the Bull Shark.

DEDICATION

This is dedicated to my partner, Josh, who has supported me endlessly the last four years both emotionally and financially. The completion of this thesis is the direct result of your love and encouragement. You got me through the high and lows of life, and I very literally wouldn't have been able to do this with you. You are my heart. I am so grateful that my path in life lead me to you. To my family, and Josh's family for their support even when they may not understand what I do. And lastly, to Rocky Ward, for giving me a chance so many years ago and introducing me to the world of population genetics.

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Contributors

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background and Significance

The Bull Shark (*Carcharhinus leucas*) is a cosmopolitan species found primarily in tropical and subtropical coastal marine and estuarine waters. Mature Bull Shark can grow up to 340 cm in length (Compagno 1984) and are predominantly found in coastal neritic (<200 m) marine environments. *C. leucas* is the only species of shark capable of tolerating long-term exposure to freshwater and has been found in both rivers and lakes connected to the sea (Thorson et al. 1966, Thorson 1971, Heupel et al. 2010, Froeschke et al. 2012). This species presence in river systems and estuaries consists primarily of juveniles or mature females (Snelson et al. 1984, Wiley and Simpfendorfer 2007, Heithaus et al. 2009, Ortega et al. 2009, Heupel et al. 2010) suggesting these habitats are predominately used for parturition and as nurseries. Litters of up to thirteen pups are born in these areas following an estimated gestation period of 10 to 11 months (Compagno 1984). Juveniles reside in their pupping grounds for multiple years, utilizing a fairly limited daily activity space of <5km (Heupel et al. 2007, Ortega et al. 2009, Heupel et al. 2010). This habitat preference is not due to any physical limitation, but rather an adaptation to avoid predators and be near abundant prey (Thorson et al. 1973, Simpfendorfer and Milward 1993, Pillans and Franklin 2004, Pillans et al. 2005, Carlson et al. 2010). While coastal habitat use does provide many advantages for *C. leucas*, it is also vulnerable to anthropogenic activities, such

pollution and habitat modification, but there has been only limited study of these impacts on this species (IUCN 2020). These impacts are often studied in congruency with overfishing while examining population trends (IUCN 2020; (Cliff and Dudley 1992, Smith et al. 1998, Dudley and Simpfendorfer 2006, O'Connell et al. 2007, Carlson et al. 2012, Froeschke et al. 2012).

Recovery capability, recruitment levels and reproductive dispersal all play critical roles in the abundance trends for a population, therefore all are key factors to consider when studying the impacts of overfishing on a species. Recovery capability refers to the maximum rate of increase for a population given its life history characteristics, hence its ability to recover or withstand overfishing. When comparing actual exploitation rates to calculated recovery rates for sharks worldwide, it was found that the exploitation rate exceeds the median recovery rate by 30%, meaning global shark trends are declining and will continue to decline until the fishing pressure is reduced (Worm et al. 2013). Large coastal species, specifically the Bull Shark, tends to have some of the lowest recovery capabilities among sharks because it is slow growing and late to mature (males: 14-15 years; females: 18 years) (Branstetter and Stiles 1987, Smith et al. 1998). These life history characteristics make them less likely to contribute to the population due to the combined effects of natural pressures and anthropogenic pressures within the coastal regions they inhabit. Sexually mature females have been reported to display reproductive philopatry (Karl et al. 2011, Tillett et al. 2012), which is a pattern for returning to a natal or postnatal nursery area to produce offspring. This high level of philopatric behavior makes the Bull Shark uniquely susceptible to localized

extinction (Hueter et al. 2005). Additionally, negative environmental impacts on these essential nursery areas may directly impact recruitment levels and lower the abundance of local populations. For instance, in South Africa, Dudley and Simpfendorfer (2006) found that significant declines in CPUE (catch per unit effort) of Bull Shark were reported after access to popular nursery habitat was blocked for extended periods of time. Each of these impacts highlight the importance of understanding population sub-structuring and patterns of gene flow for better management and protection of Bull Shark reproductive stock and their essential habitat.

Although the current status of most Bull Shark populations is unknown, general trends based on localized studies indicate that globally, the population of this species, while stable, has the potential to decline. Based upon this information, IUCN (2020) lists the Bull Shark as near threatened and likely to qualify in a threatened category upon future evaluation. While there has been significant reported declines in CPUE in parts of South Africa (Cliff and Dudley 1992, Dudley and Simpfendorfer 2006), the population status of the Western Atlantic appears to be much more stable (Carlson et al. 2012). Within the Gulf of Mexico, abundance appears to be increasing yearly (Froeschke et al. 2010, Froeschke et al. 2012) with the exception of significant localized depletions in a major estuary of Louisiana (O'Connell et al. 2007).

Despite the near threatened listing, the Bull Shark is subject to commercial and recreational harvest throughout most parts of its range. The *C. leucas* contribution to total shark landings increased from 1% between 1994-2003 (Morgan et al. 2009) to 12.6% in the Atlantic and 20.6% in the Gulf of Mexico (GOM) between 2008-2011

(Natanson et al. 2014). In addition, *C. leucas* is an apex predator for numerous marine sport fishing species, and thus play a very important role in the stability of many estuarine communities. Models predict that the loss of sharks in an ecosystem could result in complex community changes, such as trophic cascades and mesopredator release, which would in turn cause declines in commercial fish as well (Ferretti et al. 2010). In contrast, Bangley et al. (2018) found that changing environmental conditions are causing a northern expansion of estuary use in the northwestern Atlantic. The introduction of an apex predator into previously uninhabited estuaries has the potential for significant impacts on localized ecosystems. Because of their status and ecological importance, in congruency with the lack of knowledge regarding mating and movement patterns of *C. leucas* across the entire Gulf of Mexico, there is a need for determining the levels of population connectivity (i.e., gene flow) to aid in the proper design of conservation and management plans for this species.

Current Knowledge in Relation to Population Genetics

Understanding population connectivity is critical for devising an effective management plan (Hellberg et al. 2002). A comprehensive management plan takes into account many factors, such as mating behavior, dispersal of young, and movement of individuals on a fine scale rather than treating a large region as a whole. When implemented properly, a fisheries management plan will assess the finer scale movements to identify population differentiations and thus maximize the rebound potential of the species. Genetic markers are an efficient and highly informative tool

used for assessing spatial movements and gene flow within a species. Mitochondrial markers (mt) and nuclear markers differ in inheritance patterns and can be used to examine behavioral differences in sex-biased dispersal. Mitochondrial markers are strictly maternally inherited, while nuclear markers represent the contribution of both parents; therefore, genetic subdivision supported by mtDNA but not by nuclear (n) DNA may be indicative of male mediated dispersal (Portnoy et al. 2015, Momigliano et al. 2017, Pazmiño et al. 2017). Thus far, genetic analyses of Bull Shark population structure and gene flow have employed mitochondrial DNA (mtDNA) and microsatellite loci (nuclear markers). Significant differentiation among locals has been reported with mtDNA but not with microsatellites (Karl et al. 2011, Tillett et al. 2012, Laurrabaquio-A et al. 2019), and this lack of congruence has been interpreted as philopatric behavior of females toward natal sites.

MtDNA and microsatellite markers are often used in congruency, as they can be informative for answering population-level questions. However, inferences of population connectivity drawn from mtDNA are limited to investigating female patterns of gene flow as it is a maternally inherited marker, while inferences from microsatellite data can be impacted by an increase in the frequency of null alleles, variable patterns of mutation, and sparse distribution throughout the genome (Estoup et al. 2002, Putman and Carbone 2014). As an alternative, population level studies have begun to utilize single nucleotide polymorphisms (SNPs) as they are highly abundant, distributed throughout the entire genome, have relatively low error for base-calling, and have simple mutation models (Vignal et al. 2002, Brumfield et al. 2003,

Morin et al. 2004, Schlötterer 2004, Haasl and Payseur 2011). The characterization of SNPs through massive parallel sequencing is quickly becoming the preferred method for examining populations because they provide a much greater number of informative markers as opposed to previous methods, including microsatellites. Recent studies have shown that a small fraction of SNPs can be highly informative and outperform microsatellites for discerning population structure (Liu et al. 2005, Fischer et al. 2017). Due to the vast increase of informative markers, the analysis of SNPs provides the ability to increase both resolution and statistical power. Though it is still an emerging technique, SNP data has already proven useful in examining sex-biased dispersal for a coastal shark species (Portnoy et al. 2015).

This study seeks to employ next-generation sequencing to examine single nucleotide polymorphism (SNP) data, together with the characterization of the mtDNA control region (CR) to test whether the previously reported lack of differentiation with microsatellites is due to male mediated gene flow. The CR has proven to be a useful tool for assessing spatial genetic patterns of the Bull Shark at different scales. Significant structuring within mtDNA has been reported between the South China Sea and the southwestern Pacific (Deng et al. 2019), as well as between the western Indian Ocean, southwestern Pacific, and northwestern Atlantic (Pirog et al. 2019). Previous studies have found evidence for regional philopatric behavior between the Gulf of Mexico and northwestern Atlantic (Karl et al. 2012, Laurrabaquio-A et al. 2019), though no study has had representative samples across the entire Gulf of Mexico or across the newly expanded northernmost range of *C*

leucas along the northwestern Atlantic coastline (Bangley et al. 2018). This study aims to examine the genetic population structure and historical demography of representative samples from the entire basin relative to their full range along the northwestern Atlantic, and to contrast these signals against published data from the adjacent Caribbean Sea region, and from the Pacific as outliers.

Chapter Outline

The following portions of this thesis will consist of two data chapters, proceeded by a final chapter to summarize general conclusions, including results and management implications. In Chapter II, I characterized segments of the mtDNA Control Region to examine female population structure of the Bull Shark throughout the Northwestern Atlantic, in comparison to the Caribbean and Western Pacific. In order to incorporate my unique reference samples from the Caribbean, I had to trim all sequences lengths down to match the shortest reference sequence length (463 base pairs). To confirm that the population structure found was not an artifact of limited data due to shorter fragment lengths, I then ran a separate analysis from all my own samples throughout the Gulf of Mexico and Northwestern Atlantic using 733 base pairs.

In Chapter III, I measure genetic variability and assess population structure using nuclear DNA markers obtained from double digest restriction-site associate DNA (ddRAD). This method of sequencing will allow me to characterize variation across the entire genome using tens of thousands of loci, rather than a single gene as done in the previous chapter.

In Chapter IV, I will summarize the conclusions from both data chapters, including the results and implications. Broader impacts of this research are discussed with respect to management recommendations of the species.

CHAPTER II

POPULATION GENETICS AND HISTORICAL DEMOGRAPHY OF THE BULL SHARK (*CARCHARHINUS LEUCAS*) IN THE NORTHWESTERN ATLANTIC

Introduction

The Bull Shark (*Carcharhinus leucas*) is a cosmopolitan species found primarily in tropical and subtropical coastal marine and estuarine waters (< 200 m) (Compagno 1984). This species has unique physiological capabilities to tolerate long-term exposure to freshwater (Thorson et al. 1966, Thorson 1971, Heupel et al. 2010, Froeschke et al. 2012) allowing them to utilize river systems and estuarine habitats for parturition and nurseries (Snelson et al. 1984, Wiley and Simpfendorfer 2007, Heithaus et al. 2009, Ortega et al. 2009, Heupel et al. 2010). Sexually mature Bull Shark females have been reported to display reproductive philopatric behavior, by returning to their natal or postnatal nurseries to produce offspring (Karl et al. 2011, Tillett et al. 2012, Laurrabaquio-A et al. 2019). While the use of this coastal habitat does provide many advantages for *C. leucas*, it also results in increased exposure to anthropogenic activities, such as pollution, habitat degradation, and habitat modification, which makes them uniquely susceptible to localized extinctions (Hueter et al. 2005). Environmental changes of these critical nursery habitats have been shown to directly impact recruitment levels and lower the abundance of local populations (Dudley and Simpfendorfer 2006). Many of these anthropogenic impacts are often studied in conjunction with overfishing for examining population trends (IUCN 2020)(Cliff and Dudley 1992, Smith et al. 1998,

Dudley and Simpfendorfer 2006, O'Connell et al. 2007, Carlson et al. 2012, Froeschke et al. 2012).

Keys factors to consider when examining abundance trends for a population include recover capability, recruitment levels, and reproductive dispersal. The recovery capability of a species refers to the maximum rate a species can increase given life history characteristics (Smith et al. 1998). Given *C. leucas* is slow growing and late to mature (males: 14-15 years; females: 18 years) (Branstetter and Stiles 1987, Smith et al. 1998), each individual must survive the combined effects of natural and anthropogenic pressures within the coastal regions they inhabit for long periods of time in order to contribute reproductively to the population, thus making the recovery capability of the species lower compared to most teleost fishes. The dispersal capabilities of *C. leucas* have been studied directly, using satellite tagging, acoustic tagging, and mark-recapture studies to demonstrate how individuals move within their habitat. However, determining if an individual contributes to a population inhabiting a given estuary using these methods can be time consuming and expensive. An alternative is to utilize indirect methods such as genetic assessments that have been developed and employed to examine *C. leucas* reproductive philopatric behavior to a given site or region (Karl et al. 2011, Tillett et al. 2012, Laurrabaquio-A et al. 2019, Pirog et al. 2019).

Genetic markers are efficient and highly informative for assessing spatial movements and gene flow among the populations of a species. Patterns of inheritance can be quantified by measuring changes in allele frequencies resulting from evolutionary forces (mutation, migration, genetic drift, and natural selection) which can be used to

identify whether a population is subdivided (Conner and Hartl 2004). Understanding the extent to which each of these evolutionary forces is acting on a population is critical for determining population structure and creating impactful management plans of a species (Hellberg et al. 2002).

Mitochondrial markers (mt) and nuclear markers (n) differ in the mode of inheritance and can be used to evaluate sex-biased dispersal. Since mtDNA is maternally inherited, the observed population structure reflects female reproductive behavior. Previous studies have shown that mtDNA CR is informative about the spatial patterns of genetic differentiation of *C. leucas* at different scales. Significant structuring of mtDNA was reported between the South China Sea and southwestern Pacific (Deng et al. 2019), as well as between the western Indian Ocean, southwestern Pacific, and northwestern Atlantic (Pirog et al. 2019). Within our study area, comparisons of Bull Shark samples from the northern Gulf of Mexico (GoM) and parts of their range on the northwestern US coast (Karl et al. 2011, Laurrabaquio-A et al. 2019), have been carried using mitochondrial and nuclear markers, although the findings are not in agreement. Specifically, Karl et al. (2011) reported significant philopatric behavior and structuring between the northwestern Atlantic (NWA) and southwestern Atlantic, but observed no structuring between the northern GoM and NWA, whereas Laurrabaquio-A et al. (2019) reported significant structuring between the northern GoM and the NWA Bull Shark populations.

It is important to note that no study has included representative samples across the entire GoM, or across the newly expanded northernmost range of *C. leucas* along the

northwestern Atlantic coastline (Bangley et al. 2018). The southernmost region of Florida represents a cryptic barrier between the GoM and NWA for coastal shark species (Daly-Engel et al. 2012, Portnoy et al. 2015, Dimens et al. 2019). While there is not a physical barrier or physiological limitation to prevent gene flow or movement, many marine organisms appear to partition themselves between the GoM and NWA. Accordingly, this study seeks to address this potential structuring on a finer scale with the inclusion of samples from the Florida Keys.

This study focuses on characterizing genetic variability of the maternally inherited mitochondrial DNA (mtDNA) Control Region (CR) gene to test whether female *C. leucas* exhibit strong structuring towards natal sites due philopatric behavior. We aim to examine female genetic population structure and historical demography of representative samples from the entire basin relative to their full range along the NWA coast of the US. To gain a better understanding of the timing and relative degrees of differentiation between these two regions, we will contrast the observed signals against published data from the adjacent Caribbean Sea region, as well as from Pacific samples as outliers. We hypothesis that historical demography patterns will show that the Caribbean population gave rise to its northern counterparts, resulting in the recent expansion in to the GoM.

Materials and Methods

Sample collection

Bull Shark tissue samples (n=167), consisting of fin clips or biopsy punches, were obtained opportunistically from fishing charters, and from studies or surveys for scientific purposes throughout the Gulf of Mexico (GoM), and along the Northwestern coastline of the United States. In the GoM, samples came from Campeche, Mexico (n = 26), Texas (n = 26), Louisiana (n = 27), the Big Bend Region in Florida (NW FL, n = 15), near Everglades National Park (SW FL, n = 15), and the Florida Keys (Keys, n = 9). On the Atlantic coastline, samples came from the Indian River Lagoon in Florida (SE FL, n = 21), along the Georgia/South Carolina coastline (Mid Atlantic, n = 13), and from North Carolina (n = 15). Samples were collected and immediately preserved in 99% ethanol and stored at room temperature, or frozen at -20°C, until assayed (Figure 2-1). Additional published Control Region (CR) reference sequences were included in a secondary analysis, comprising sequences from Australia (Western Pacific, n = 166), the China Straight (W Pacific, n = 1), Teacapan, Mexico (E Pacific, n = 2), and the Caribbean Sea (Nicaragua, n = 6). A total of 334 Bull Shark mtDNA CR sequences were analyzed (Table A-1).

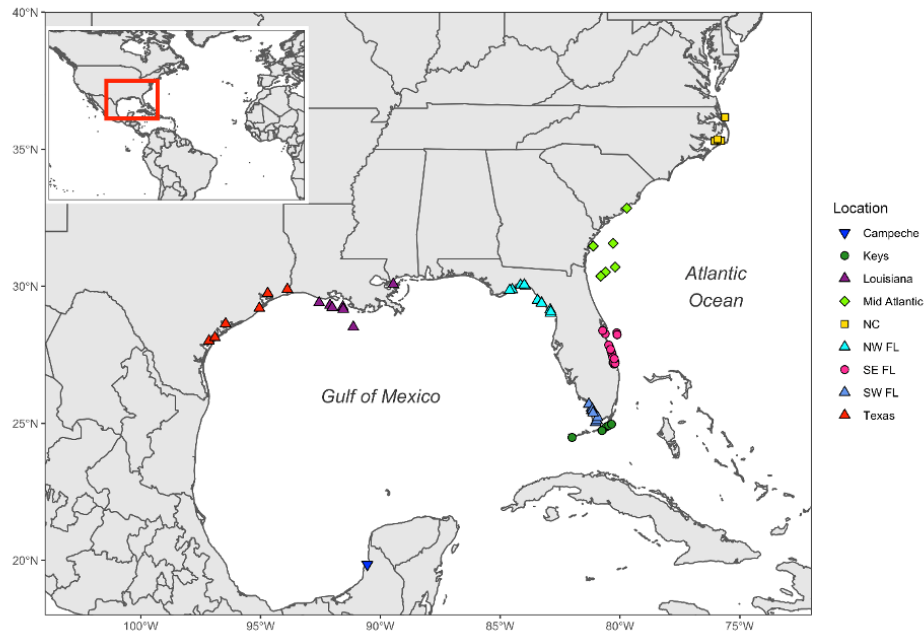

Figure 2-1. Sample map of the northwestern Atlantic coastline. Point color cooresponds to sampling locality, and point shape reflects best AMOVA grouping.

DNA Isolation

High yields of high molecular weight DNA were obtained using the Zymo *Quick*-DNA Universal Kit following the manufacturer's protocols for tissue extraction (Zymo Research, Irvine, CA, U.S.A.). Isolated DNA was stored at 4°C until assayed. The quality of isolated DNA was visualized by running 4 µl in a 1% ethidium bromide (EtBr) stained tris-acetate (TA) gel and quantified using both Nanodrop and Qubit instruments (Thermo Fisher Scientific, Waltham, MA).

PCR amplification and Sequencing

Polymerase chain reactions (PCR) were performed using 1µL of the template DNA. Initial attempts to amplify and sequence the mtDNA Control Region (CR) as described in Tillett et al. (2012) gave mixed results characterized by a failure to obtain sequences using their reverse primer. Accordingly, the mtDNA CR was amplified using the forward primer GWF (5' CTGCCCTTGGCTCCCAAAGC) (Pardini et al. 2001) and a new reverse primer CLEU-R (5' TATAGGAGGTTTCTTTCCGAGAG) developed using the Primer 3 software (Koressaar and Remm 2007, Untergasser et al. 2012) in Geneious Prime v2019.1.8 (Biomatters Ltd, Aukland, NZ), based on partial *C. leucas* CR sequences that were generated with the forward primer. DNA amplification was carried out in a Roche LightCycler 96 (Roche Diagnostics). Polymerase chain reactions were prepared in 10.0 µL volumes, consisting of 5.0 µL 2X EconoTaq Plus Master Mix (Lucigen), 0.5 µL LC Green (Idaho Technologies), 0.2 µM GWF, 0.2 µM CLEU-R, 2.5 µL ddH$_2$O, and 1.0 µL of the template DNA. Thermocycling conditions were as follows; an initial denaturing step at 94°C for 90s followed by 35 cycles of denaturing at 94°C for 10s, annealing at 59°C for 30s, and extension at 72°C for 60s, followed by a final extension step at 72°C for 5min. Negative controls were included in all reactions. Amplification curves were visualized in real time on the LightCycler96, and PCR products were then visualized via electrophoresis on a 2% TA agarose gel pre-stained with EtBr run at 100 mV for 35 minutes. PCR products with a single band were diluted 1:10 for post-PCR cleanup and then sequenced in each direction using the ABI BigDye terminator v3.1 (Applied Biosystems) following the fast cycle sequencing protocol (Platt

et al. 2007). Cycle sequencing products were cleaned using the ZR DNA Sequencing Clean-up Kit (Zymo Research), and Sanger sequencing was performed in a 3130 Genetic Analyzer (Applied Biosystems; www.appliedbiosymstems.com).

Population Structure Analysis

Multiple sequence alignments of the mtDNA CR of every individual (n=167) captured in the NW Atlantic and the Gulf of Mexico were carried out in Geneious Prime v2019.1.8 (Biomatters Ltd, Aukland, NZ) using the progressive pairwise alignment algorithm. Each polymorphic site was confirmed by inspecting the corresponding nucleotide in the original electropherograms. Haplotype data files were generated using DnaSP v6.12.03 (Rozas et al. 2017).

Arlequin v3.5 (Excoffier and Lischer 2010) was used to estimate genetic variation within and between populations with an analyses of molecular variance (AMOVA**),** and to estimate descriptive statistics, including the number of haplotypes (M), haplotypic diversity (*h*), pairwise differences between haplotypes, nucleotide diversity (π), and the number of polymorphic or segregating sites (S) using a corrected Tamura and Nei model (Tamura and Nei 1993). When conducting phylogenetic analyses in MEGA v7.0.21 (Kumar et al. 2016) and POPART (Leigh and Bryant 2015) insertions and deletions (indels), which occur at about the same frequency as transversions, were weighted the same to adjust for program biases against informative indels (Alvarado-Bremer et al. 1995). JModelTest2 (Guindon and Gascuel 2003, Darriba et al. 2012) was

used to select the best fit model of nucleotide substitutions and its associated gamma distribution based on BIC criterion. A single representative of each haplotype was randomly selected to generate a haplotype tree. A Maximum Likelihood (ML) tree was constructed in MEGA from transitions (Ts) and transversions (Tv) with 1000 bootstraps using a gamma corrected Tamura and Nei model (Tamura and Nei 1993). For comparative purposes, a Minimum Evolution (ME) tree was constructed using the p-distance model using Tv + Ts with 1000 bootstrap replicates. A Minimum Spanning Network (MSN) in POPART (Leigh and Bryant 2015) was constructed to simplify the visualization of the phylogeographic association of haplotypes. A Mantel test of isolation by distance (IBD) was calculated using Slatkin's Linearized F_{ST} in relation to pairwise distance between each sampling locality, computed using geographic distance in kilometers measured as the shortest coastal line between localities (Slatkin 1995). A Salicru X^2 test (Salicru et al. 1993) was used to test significance of pairwise differences since distribution of diversity statistics have an asymptotic distribution.

Reference Sample Population Structure Analysis

To further examine the phylogenetic origin of haplotypes within the sampling region, we included reference Bull Shark mtDNA CR sequences from previous studies (Kitamura et al. 1996, Tillett et al. 2012, Chen et al. 2014). However, this analysis was conducted separately because substantial trimming of some of the CR sequences was required when optimizing the multiple sequence alignment because of the different sets

of PCR primers used in respective studies. JModelTest2 BIC criterion revealed the best model for the trimmed dataset to be a Tamura-Nei + I corrected model for construction of haplotype trees. Population structure analyses followed the same protocols as described above.

The levels of neutral genetic variation in a population are informative of long-term female effective population size [$N_{e(f)}$] can be estimated from the relation between the levels of neutral genetic variation measure of genetic diversity θ, and the long-term effective female population size [$N_{e(f)}$] is $\theta = 2N_{e(f)}\mu$, where μ is the substitution rate per generation, as described in Roman and Palumbi (2003). In order to examine historical demography, rate of divergence was calculated using randomly selected representatives from the Gulf of Mexico that were then compared to samples from the eastern Pacific within DnaSP v6.12.03 (Rozas et al. 2017) to obtain Tamura-Nei distances (D_A) between the populations**.** A generation time of 18 years was used based on estimates for sexual maturity of females (Branstetter and Stiles 1987). Mutation rates were calculated based on time since divergence from a known geological event, the emergence of the Isthmus of Panama, estimated to have occurred approximately 3.2 million years ago (Mya) (O'Dea et al. 2016) given we have representative samples from either side of the land formation. Pairwise mismatch distributions (Rogers and Harpending 1992), divergence between groups (D_A statistic) (Nei 1987), population neutrality tests (R2 statistic) (Ramos-Onsins and Rozas 2002), estimated mutational time, and tau (τ) (Rogers and Harpending 1992) were generated to estimate patterns of historical demography in relation to population expansion.

Results

Gulf of Mexico versus northwestern Atlantic Coast of United States

A total 733 base pairs (bp) of mtDNA CR sequence was obtained for 167 individual Bull Shark (*C. leucas*) collected in the NW Atlantic and GoM, containing a total of 14 polymorphic sites, including three indels, seven transitions, and four transversions, that defined 21 distinct haplotypes. Analyses of these CR sequences revealed a low level of nucleotide sequence diversity (π) of 0.001206 ± 0.000982 and a haplotypic diversity (h) of 0.6234 ± 0.1034 within and among these two groups (Table 2-1). There were significant differences in the levels of haplotypic diversity among the samples, with Louisiana and NWFL displaying significantly less variation ($h < 0.36$) than SWFL, the Keys, SEFL, the Mid Atlantic and NC ($p < 0.05$; Table 2-2). The Keys was the most variable sample ($h = 0.86$) characterized in this study, except for SEFL, which contained similar levels of variation ($h = 0.78$).

Comparison of pairwise F_{ST} values identify the Florida Keys, SE FL, and the Mid Atlantic as not different from one another, but individually significantly different from Campeche ($p < 0.05$), Louisiana, and SW FL ($p < 0.01$). Linearized F_{ST} calculations of Slatkin's distance and Reynold's distance (Table 2-3) yielded similar relationships. Despite the apparent differences between the GoM and Atlantic samples, a Mantel Test failed to support IBD (R2 = -0.09935, p = 0.6646) (Figure 2-2).

Location	*n*	M	*h*(SD)	*π* (SD)	S	Ts	Tv	I
1. Campeche	26	6	0.5662 (0.1085)	0.001845 (0.001316)	6	5	1	0
2. Texas	26	4	0.6123 (0.0806)	0.001156 (0.000945)	2	2	0	0
3. Louisiana	27	3	0.3305 (0.1083)	0.000548 (0.000584)	2	2	0	0
4. SW FL	15	5	0.6762 (0.1049)	0.000417 (0.000512)	4	2	0	2
5. NW FL	15	3	0.3619 (0.1448)	0.000383 (0.000487)	2	1	0	1
6. Keys FL	9	5	0.8611 (0.0872)	0.001576 (0.001270)	4	2	1	1
7. Mid Atlantic	13	7	0.7308 (0.1332)	0.002064 (0.001490)	6	3	2	1
8. SE FL	21	7	0.7857 (0.0635)	0.001492 (0.001138)	7	3	2	2
9. North Carolina	15	4	0.6857 (0.1040)	0.001377 (0.001099)	3	3	0	0
All Samples	**167**	**21**	**0.6234 (0.1034)**	**0.001206 (0.000982)**	**14**	**7**	**4**	**3**

Table 2-1. Molecular indices for 733 bp of mtDNA sequences of the CR for C. leucas by sampling location. Table shows number of haplotypes (M), haplotypic diversity (h), nucleotide diversity (π), standard deviation (SD), number of segregating (polymorphic) sites (S), number of transitions (Ts), number of transversions (Tv), and number of indels (insertions and/or deletions, I).

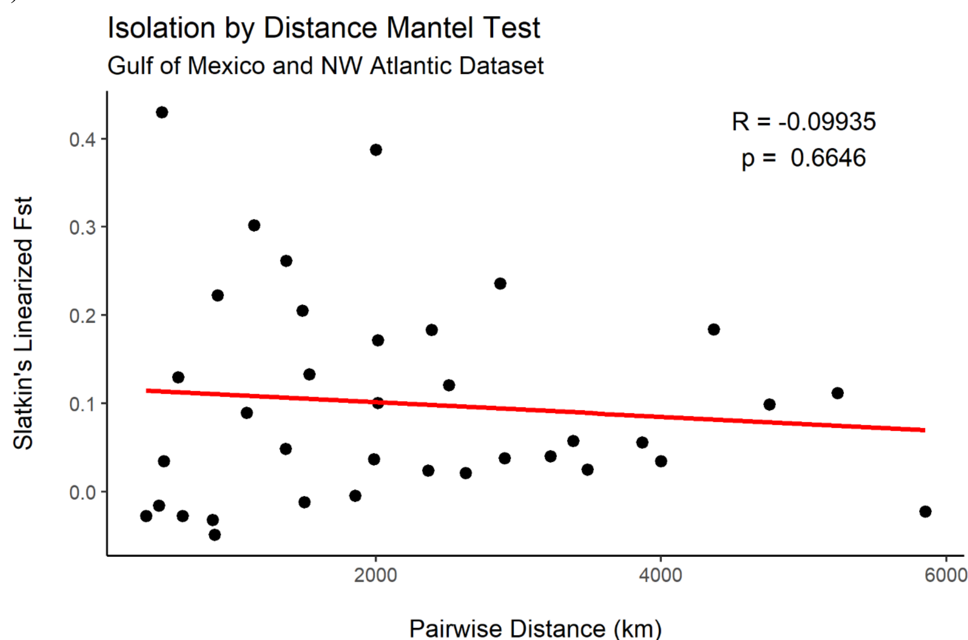

Figure 2-2. Pairwise distances (km) measured along the coastline between sampling locations plotted against pairwise values of Slatkin's linearized FST for 733 bp of mtDNA Control Region sequences for C. leucas in the Northwest Atlantic.

	MX	TX	LA	SWFL	NWFL	KEYS	MA	SEFL	NC
MX		0.34107	-1.5375	-0.7289	1.1291	**-2.1186**	-0.9581	**-1.7460**	-0.7951
TX	-0.0050		**2.0874**	-0.4830	1.5110	**-2.0953**	-0.7611	**-1.6899**	-0.5579
LA	0.0234	0.0336		**-2.2928**	-0.1737	**-3.8161***	**-2.3318***	**-3.6258***	**-2.3656***
SWFL	0.0529	0.0912	-0.0119		**-1.7578**	-1.3555	-0.3220	-0.8930	-0.0643
NWFL	0.0387	0.0460	-0.0332	-0.0284		**-2.9533**	**-1.8750**	**-2.6804**	**-1.8163**
KEYS	**0.1553**	0.1077	**0.2793***	**0.3008**	**0.23189**		0.8184	0.6990	1.2924
MA	**0.1004**	0.0541	**0.1909***	**0.2073***	**0.1464**	-0.0514		0.3720	0.2669
SEFL	**0.0901**	0.0364	**0.1548***	**0.1819***	0.1172	-0.0285	-0.0164		0.8207
NC	-0.0230	0.0332	0.0242	0.0353	0.0206	**0.1701**	**0.1146**	0.0821	

Table 2-2. Values for pairwise comparisons for 733 bp of CR for Bull Shark in the northwestern Atlantic. Z-scores from Salicru χ^2 test for pairwise comparisons of haplotypic diversity are above the diagonal. Pairwise F_{ST} are below the diagonal. Values with a significance at $p < 0.05$ are denoted in bold, and significance at $p < 0.01$ denoted in bold with *. MX, Campeche Mexico; TX, Texas; LA, Louisiana; SWFL, SW Florida; NWFL, NW Florida; KEYS, Keys Florida; MA, Mid-Atlantic; SEFL, SE Florida; NC, North Carolina.

	MX	TX	LA	SWFL	NWFL	KEYS	MA	SEFL	NC
MX		0.00000	0.02370	0.05436	0.03946	0.16871	0.10577	0.09440	0.00000
TX	0.00000		0.03412	0.09559	0.04705	0.11399	0.05560	0.03704	0.03381
LA	0.02398	0.03471		0.00000	0.00000	0.32759	0.21186	0.16819	0.02455
SWFL	0.05586	0.10031	0.00000		0.00000	0.35785	0.23237	0.20082	0.03595
NWFL	0.04025	0.04817	0.00000	0.00000		0.26382	0.15830	0.12465	0.02081
KEYS	0.18378	0.12074	0.38762	0.43026	0.30190		0.00000	0.00000	0.18642
MA	0.11157	0.05717	0.23598	0.26158	0.17152	0.00000		0.00000	0.12175
SEFL	0.09900	0.03774	0.18316	0.22240	0.13275	0.00000	0.00000		0.08575
NC	0.00000	0.03439	0.02485	0.03661	0.02103	0.20492	0.12947	0.08954	

Table 2-3. Pairwise population comparisons for 733 bp of mtDNA Control Region sequences for Bull Shark in the Northwest Atlantic. Slatkin's linearized F_{ST} is below the diagonal, and Reynold's Distance is above the diagonal. MX, Campeche Mexico; TX, Texas; LA, Louisiana; SWFL, SW Florida; NWFL, NW Florida; KEYS, Keys Florida; MA, Mid-Atlantic; SEFL, SE Florida; NC, North Carolina.

AMOVA most optimal phylogeographic grouping occurred when samples were allocated into three regional groups, consisting of a GoM grouping (1: Campeche, Texas, Louisiana, SW Florida, NW Florida), a Florida Keys grouping (2, Keys), and an Atlantic grouping (3: SE Florida, Mid-Atlantic, North Carolina). Although most of the variance (90.1%) was contained within samples, a relatively large proportion of the variance (6.5%) was due to differences among groups (F_{CT}, $p < 0.05$), with the remaining variance (3.5%) explained by differences among-samples within-groups (F_{SC}, $p < 0.05$; Table 2-4**)**. The Florida Keys samples were originally expected to group within the Gulf of Mexico samples, but the high level of haplotypic diversity within that region suggests that the Keys may be a mixing zone between the GoM and Atlantic populations, and as such it would be inappropriate to assign it to either one. Accordingly, from here on these populations will be referred to hereafter as GoM, Keys, and Atlantic.

The haplotypic relationships within and between populations (h = 21) can be visualized using an MSN that shows patterns of phylogeographic structuring between the GoM and Atlantic (Figure 2-3). It is important to note that due to the overall low levels of nucleotide diversity, the GoM and Atlantic are only separated by one to two mutational steps (denoted by hash marks). A dominate haplotype was identified within the GoM, representing 67.89% of the individuals in this region. but found a much lower frequency among the Atlantic samples with only 4.08% of the individuals expressing this haplotype (H1). While the Atlantic samples show higher levels of haplotypic diversity than the GoM, one haplotype (H8) was identified as the centroid of a star phylogeny that contains most (34.69%) of the haplotypes sampled in this region, with

nine of them separated from the ancestral haplotype by a single mutation (Fig. 2-3), and include one haplotype (H11) from SW Florida. Lastly, the high haplotypic diversity of the Keys (Tables 2-1 and 2-2) can be visualized by their apparent equal mixing between dominate haplotypes of both the GoM and Atlantic. To further illustrate haplotypic relationships within sampling localities, a ME tree was generated using a single representative of each haplotype, with 1000 permutations. Unique haplotypes are shown at the tips of the branches, with different symbols corresponding to specific locations where the specific haplotype was found, along with the corresponding number of individuals within each locality containing the corresponding haplotype (Figure 2-3). The majority of representative samples within each sampling locality of the GoM, ranging from 53.3%-80% respectively, express the dominate haplotype H1.

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Variance Components**	**Percentage of Variation**
Among Groups	2	3.860	0.03107 Va	6.46
Among Populations Within Groups	6	4.566	0.01671 Vb	3.47
Within Populations	158	68.428	0.43309 Vc	90.07
Total	166	76.854	0.48086	
Fixation Indices			**P-values (≥)**	
	F_{SC}:	0.03714	0.00050 +/- 0.00022	
	F_{ST}:	0.09935	0.02772 +/- 0.00176	
	F_{CT}:	0.06461	0.02515 +/- 0.00146	

Table 2-4. AMOVA results for 733 bp of mtDNA Control Region sequences for Bull Shark in the Northwest Atlantic. Population 1 (GoM): Campeche-Mexico, Texas, Louisiana, SW Florida, NW Florida; Population 2: Keys-Florida; Population 3 (NW Atlantic): SE Florida, Mid-Atlantic, North Carolina. P-values for fixation indices are based on significance tests with 10,100 permutations.

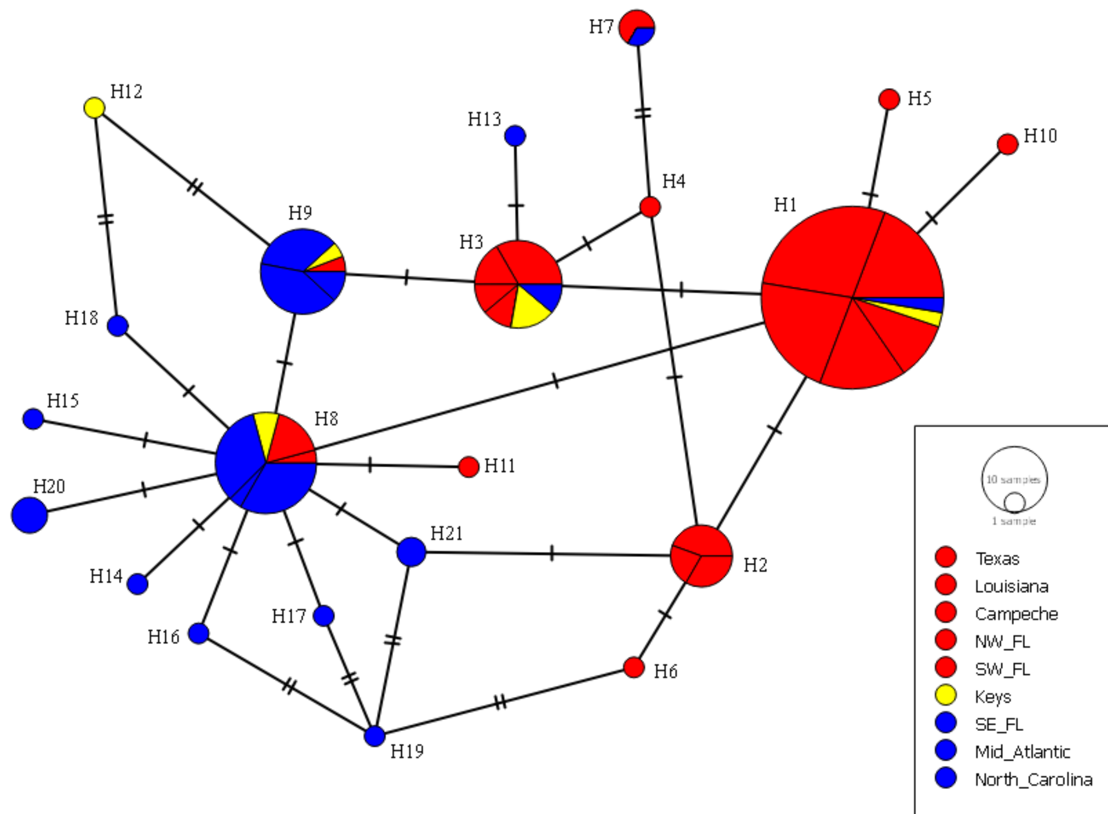

Figure 2-3. Minimum spanning network (MSN) for 733 bp of mtDNA Control Region sequences for Bull Shark in the Northwest Atlantic. Circles correspond to a haplotype, with size indicative of the number of individuals within each haplotype. Color groupings correspond to the best AMOVA groupings. Blue corresponds to individuals sampled within the Gulf of Mexico, yellow corresponds to individuals sampled in the Keys, and red corresponds to individuals sampled on the Atlantic Coast. Hash marks indicate genetic distance in the form of segregating sites between each haplotype.

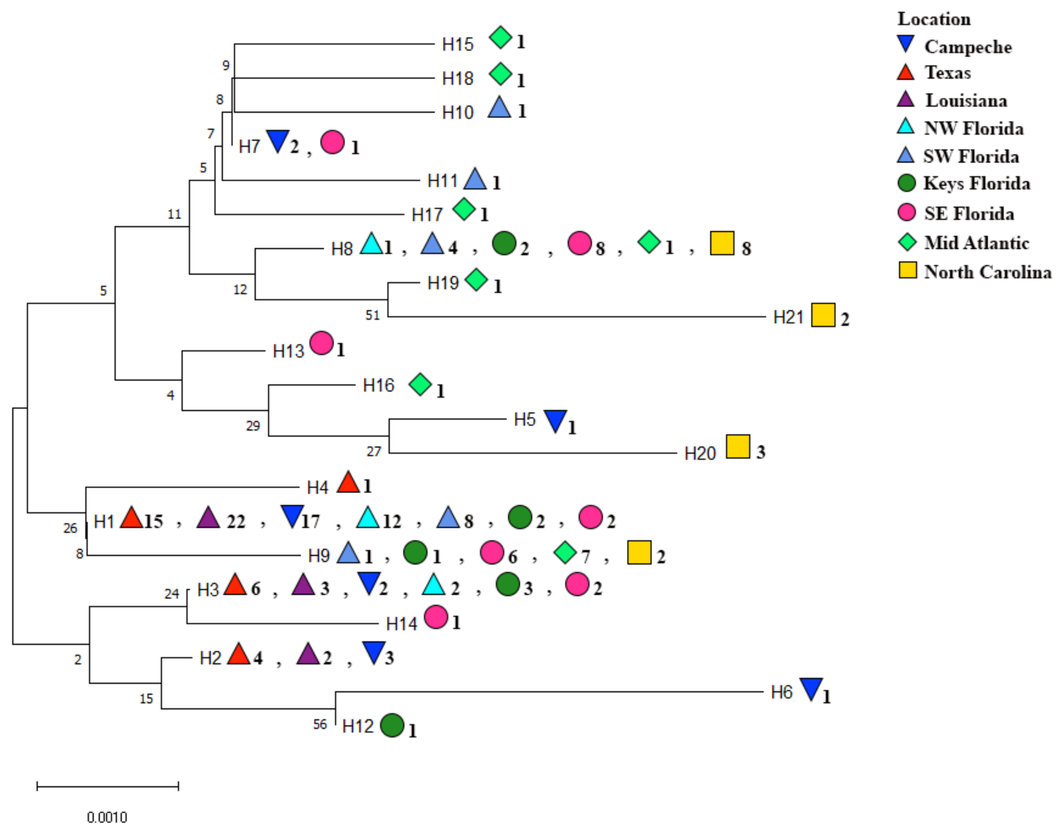

Figure 2-4. Haplotype Minimum Evolution (ME) tree rooted at midpoint for 733 bp of mtDNA Control Region sequences of Bull Shark from the Northwest Atlantic and Gulf of Mexico. Symbols correspond to sampling locations, with numbers indicative of the number of individuals within each haplotype from said locality. Bootstrap-values are shown at each node and are based on 1000 permutations.

Northwestern Atlantic vs Caribbean vs Pacific

Reference sequences (n = 166) from previous studies (Table A-1) were added to the original dataset (n = 167) and trimmed to 463bp of mtDNA CR sequence for 347 Bull Shark individuals. Analyses of these CR sequences resolved more haplotypes (M=26), and more polymorphic sites (S=23, Table 2-5) than the NWA analyses. An overall level of haplotype diversity (h) of 0.5208 ± 0.0909 and a nucleotide diversity (π) of 0.00156 ± 0.001332 was obtained. With the exception of both Nicaragua (h = 0.933 ± 0.1217) and the Florida Keys (h = 0.8889 ± 0.0910), most sampling localities contained relatively low levels of haplotype diversity as the original analyses. The lowest haplotype diversity was reported for the Eastern Pacific, where a single haplotype private to that location was found (h = 0.0000 ± 0.0000).

Location	*n*	M	*h*(SD)	*π* (SD)	S	Ts	Tv	I
Texas	26	2	0.4092 (0.0832)	0.000890 (0.000940)	1	1	0	0
Louisiana	27	2	0.2051 (0.0947)	0.000446 (0.000628)	1	1	0	0
Campeche	27	6	0.5014 (0.1119)	0.001314 (0.001198)	5	4	0	0
NW FL	15	3	0.4571 (0.1406)	0.000538 (0.000720)	2	1	0	1
SW FL	15	3	0.5619 (0.0954)	0.000290 (0.000511)	2	1	0	1
Keys FL	9	6	0.8889 (0.0910)	0.001997 (0.001716)	3	3	0	1
SE FL	21	6	0.7476 (0.0692)	0.001349 (0.001232)	4	2	0	2
Mid Atlantic	13	4	0.6795 (0.1116)	0.001791 (0.001534)	3	2	0	1
North Carolina	15	3	0.5333 (0.1259)	0.001288 (0.001221)	2	2	0	0
W Pacific	171	9	0.3324 (0.0458)	0.001487 (0.001258)	9	7	1	1
E Pacific	2	1	0.0000 (0.0000)	0.000000 (0.000000)	0	0	0	0
Nicaragua	6	5	0.9333 (0.1217)	0.007330 (0.005031)	8	5	2	1
All Samples	**347**	**26**	**0.5208 (0.0909)**	**0.001560 (0.001332)**	**23**	**17**	**3**	**3**

Table 2-5. Molecular indices of variation obtained from trimmed (463 bp) mtDNA CR of *C. leucas* for each sampling location. M, number of haplotypes; *h,* haplotype diversity; *π*, nucleotide diversity; SD, standard deviation; S, number of segregating (polymorphic) sites; Ts, number of transitions; Tv, number of transversions; I, number of indels (insertions and/or deletions).

Despite the lower levels of haplotype diversity, the phylogeographic association of genetic differentiation was sufficient to resolve significant AMOVA groupings between (1) the GoM (Campeche; Texas; Louisiana; NW FL; SW FL), (2) Atlantic (Keys; SE FL; Mid Atlantic; North Carolina), (3) Caribbean (Nicaragua), (4) Eastern Pacific (Teacapan – Mexico), and (5) Western Pacific (Australia; China Straight), respectively (Table 3-6). AMOVA results indicated significant variance among groups relative to the variance of the full dataset (F_{CT}; $p < 0.01$). The very high percentage of variation between groups (85.6%) is likely due to the inclusion of the Western Pacific samples.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	4	413.981	1.88966 Va	85.61
Among Populations Within Groups	7	2.899	0.00555 Vb	0.25
Within Populations	335	104.548	0.31208 Vc	14.14
Total	166	76.854	0.48086	
Fixation Indices			**P-values (≥)**	
	F_{SC}:	0.01748	0.00000 +/- 0.00000	
	F_{ST}:	0.85861	0.08337 +/- 0.00292	
	F_{CT}:	0.85610	0.00000 +/- 0.00000	

Table 2-6. AMOVA results for 463 bp of mtDNA Control Region sequences for Bull Shark. Population 1 (GoM): Campeche-Mexico, Texas, Louisiana, SW Florida, NW Florida; Population 2 (Atlantic): Keys-Florida, SE Florida, Mid-Atlantic, North Carolina; Population 3 (E Pacific): Teacapan-Mexico; Population 4 (W Pacific): Australia, China-Straight; Population 5 (Caribbean): Nicaragua. P-values for fixation indices are based on significance tests with 10100 permutations.

The haplotypic relationships within and between populations (M = 26; Table 2-5) were visualized using an MSN to show the phylogeographic structuring between the

GoM, Atlantic, Caribbean, eastern Pacific, and western Pacific (Figure 2-5). The western Pacific showed a similar pattern of a single dominant haplotype within that region, accounting for > 81% of the individuals, with an additional eight haplotypes private to that region. There were no shared haplotypes across the Pacific Ocean, nor between the Atlantic and Pacific basins, in spite of the relatively short genetic distance (four mutations) separating the western Pacific from the northwestern Atlantic lineages. The MSN shows the major western Pacific lineage (H2) separated by four mutational steps from a northwestern Atlantic haplotype (H12). Interestingly, the eastern Pacific lineage (H16) is an additional five mutational steps away from the NWA haplotype (H12), for a total of nine mutational steps separating the eastern Pacific and western Pacific lineages. The samples from the Caribbean Sea (Nicaragua) showed a higher value of haplotypic diversity, and contained multiple haplotypes that were four mutational steps apart from the haplotypes in the northwestern Atlantic, although one haplotype (H12) was shared, and another (H25) was more closely grouped to the Atlantic lineages than to the divergent cluster of Caribbean linages (Figure 2-5). The northwestern Atlantic and western Pacific coincide in the presence of dominant haplotypes giving rise to many rare haplotypes, as shown by the star-like phylogeny patterns surround haplotype H2, H10, and H11. To further illustrate haplotypic relationships within sampling localities, a ME tree was constructed using a single representative of each haplotype, with 1000 permutations. A ML tree was generated using a Tamura-Nei + I correction model to add additional bootstrap (bs) values for branches with moderate support >45 (Figure 2-6). Both the ME and ML tree gave support (bs > 55) for a separate clade of the western

Pacific population. Notably, most of the Caribbean samples from Nicaragua group in a well-supported clade which received the highest support (bs> 80), of any of the northwestern Atlantic or Pacific clades. This divergent group is clustered together within the MSN (Figure 2-5) as well, though shown four mutational steps from a haplotype (H15) found in both the Gulf of Mexico and the NWA. Despite the apparent phylogeographic association, a Mantel test failed to support IBD ($R2 = 0.4404$, $p = 0.07$; Figure 2-7).

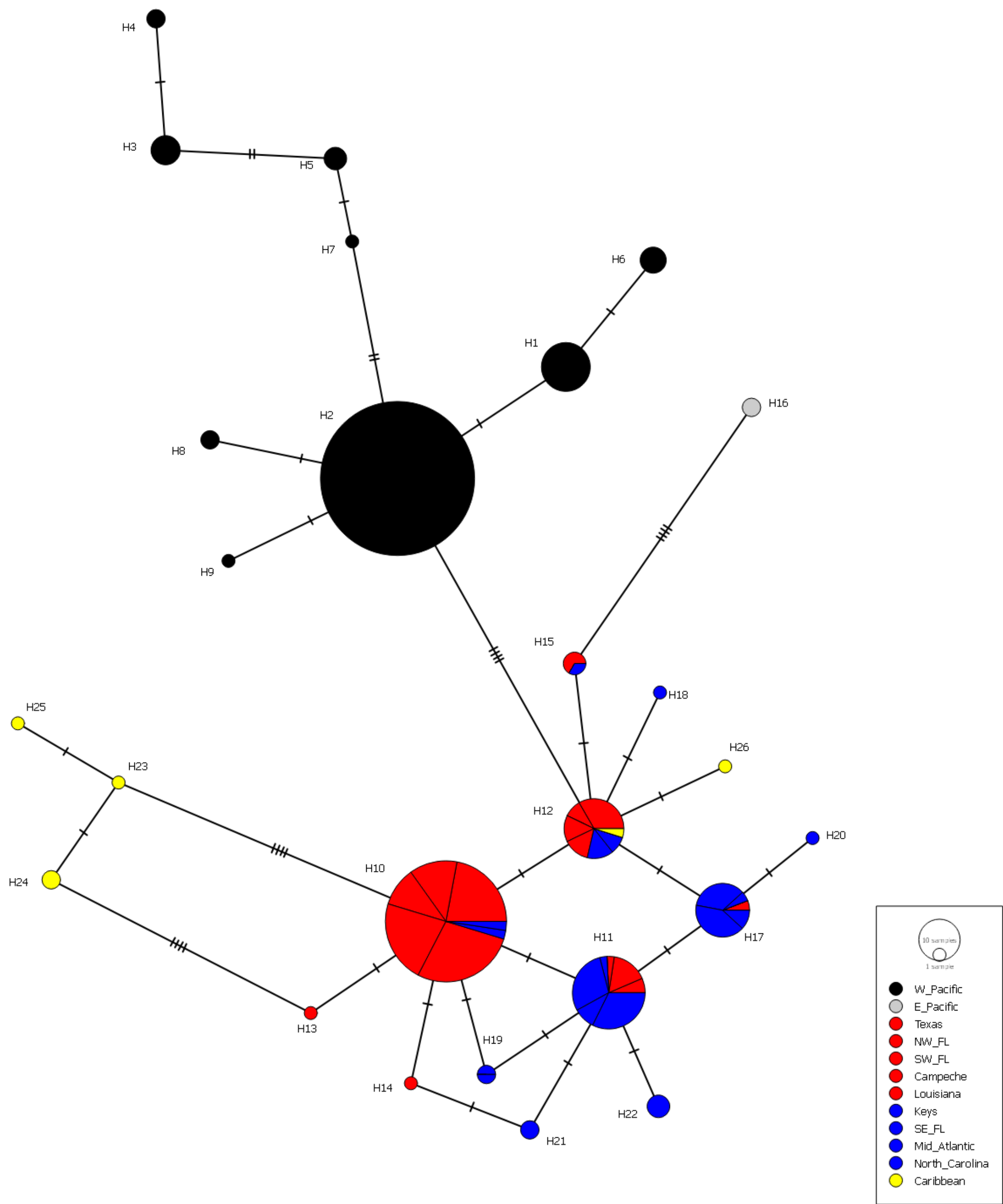

Figure 2-5. Minimum spanning network (MSN) for 463 bp of mtDNA Control Region sequences for Bull Shark. Circles correspond to a haplotype, with size indicative of the number of individuals within each haplotype. Color groupings correspond to the best AMOVA grouping. Blue correspond to individuals sampled within the Gulf of Mexico, yellow corresponds to individuals sampled in the Caribbean, red corresponds to individuals sampled on the Atlantic Coast, black corresponds to individuals from the western pacific, and grey corresponds to individuals from the eastern Pacific. Hash marks indicate genetic distance in the form of segregating sites between each haplotype.

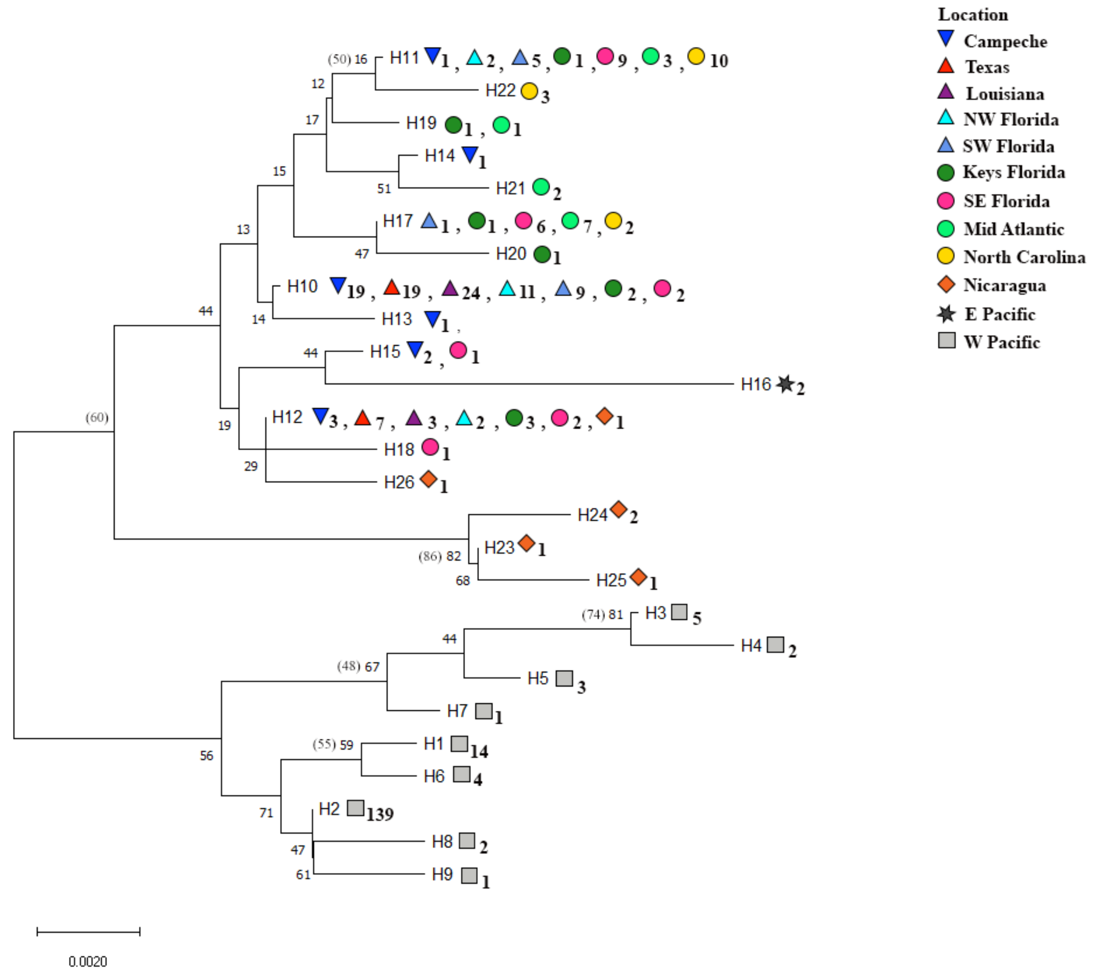

Figure 2-6. Haplotype Minimum Evolution (ME) tree for 463 bp of mtDNA Control Region sequences for Bull Shark. Symbols shapes correspond to AMOVA grouping (Triangle: GoM, Circle: Atlantic, Diamond: Caribbean, Star: E Pacific, Square: W Pacific), with numbers indicative of the number of individuals within each haplotype from said locality. Bootstrap-values are shown at each node and are based on 1000 permutations. Corresponding bootstrap values (>45) from a Maximum Likelihood tree are indicated within parenthesis and are based on 1000 permutations.

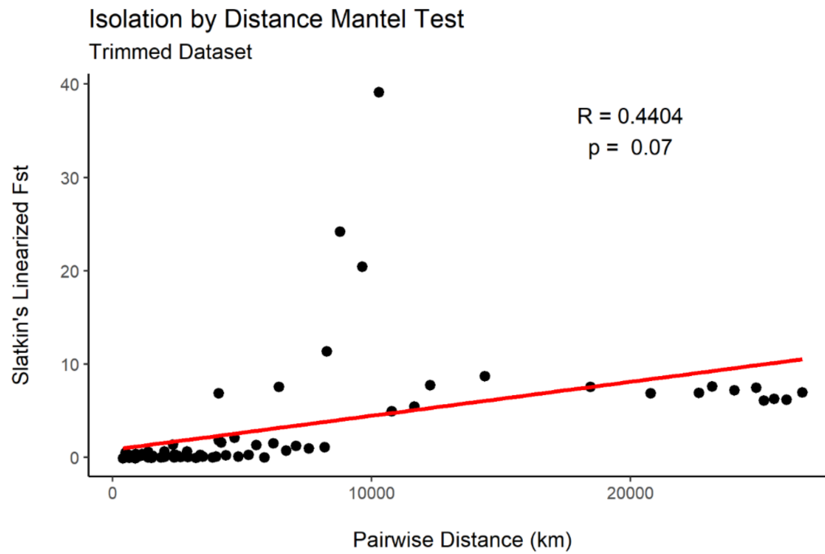

Figure 2-7. Pairwise distances (km) measured along the coastline between sampling locations plotted against pairwise values of Slatkin's linearized F_{ST} for 463 bp of mtDNA Control Region sequences for Bull Shark.

The historical demographic signature of Bull Shark was nearly identical between both regions. Both Tajima's D statistic and the R2 statistic were not significant, suggesting the observed genetic patterns are not due to recent expansion (**Table 2-7**).

Population	D_A	τ	D (P)	R2 (P)	T	N_e
GoM	0.01011	0.551	-1.05433 (0.139)	0.0924 (0.490)	3.2	105.5
Atlantic	0.00914	1.313	-0.74065 (0.290)	0.0924 (0.411)	3.2	194.5

Table 2-7. Historical population demography parameters and estimates of female effective population size (N_e) for populations of Bull Shark in the Gulf of Mexico. D_A, Tamura-Nei genetic distance between each population-pair in AMOVA grouping against the E Pacific population; τ, estimated mutational time since population expansion; D, Tajima's D with probability value (P); R2, Ramos-Onsins and Rozas's R2 with probability value (P); T, time since divergence in millions of years used for mutation rate estimations; N_e, estimated female effective population size, in thousands of individuals.

Discussion

Gulf of Mexico versus northwestern Atlantic Coast of United States

Reproductive philopatry is a behavior that restricts gene flow between regions despite a lack of physical barriers. This behavior has been observed in across many species of sharks, including female Bull Shark (Hueter et al. 2005, Tillett et al. 2012, Portnoy et al. 2015). Identification of cryptic barriers of gene flow, such as this, can be difficult to identify but is important for understanding population dynamics for fisheries management. The extent to which female reproductive philopatry is occurring can be studied by quantifying genetic relatedness of maternally inherited mtDNA. This study was aimed at comparing levels of genetic variation of a large coastal shark, *C. leucas*, from representative samples across the entire range of the northwestern Atlantic to identify structuring on a fine scale.

Although the Bull Shark is a fairly common species with widespread geographic distributions, relatively low genetic estimates of haplotype and nucleotide diversity were observed within the mtDNA CR particularly in the northern Gulf of Mexico, from Louisiana to Northwest Florida. These observed values are similar to those reported in previously studies (Karl et al. 2011, Deng et al. 2019, Pirog et al. 2019). Despite these low estimates, nearly 6.5% of the total genetic variation was explained by genetic differentiation among the GoM, Keys, and Atlantic groups (Table 2-4). While the observed structuring between the GoM and Atlantic are in general agreement with Laurrabaquio-A et al. (2019), it conflicts the panmictic reports described in Karl et al.

(2011). Genetic breaks associated with south Florida are well described across many taxa, including multiple coastal shark species (Daly-Engel et al. 2012, Portnoy et al. 2015, Dimens et al. 2019). Despite the lack of geographic barriers in place, our results indicate that southern Florida may not only act as an effective cryptic barrier to gene flow in *C. leucas*, but may potentially be a mixing zone between the GoM and NWA given the high haplotype diversity of the Keys that is 1.7 times higher than the mean haplotype diversity across samples.

While we found evidence for phylogeographic associations of the mtDNA CR between the GoM and Atlantic, the AMOVA and pairwise F_{ST} values (Table 2-4; Table 2-2) indicate little to no structuring exists within each grouping. This is not surprising given the sample sizes, but also because F_{ST} was an index that was not originally intended to be applied to molecular data. Instead, ϕst analogs, calculated within Arlequin to measure genetic differentiation, better summarize the patterns of structuring, particularly when levels of haplotypic diversity are high (Excoffier et al. 1992). Both mean haplotype diversity (0.6234 ± 0.1034) and mean nucleotide diversity (0.001206 ± 0.000982) across samples were extremely low (Table 2-1); However, we observed significant differences in haplotype diversity between localities as shown by the Salicru test (Table 2-2). In general, haplotype diversity within the GoM (1-5; Table 2-2) was significantly lower than the Keys (6), and the NWA (7-9).

Given the expectation of strong philopatric behavior of *C. leucas* to natal sites, our results suggest the Gulf of Mexico may act as a singular natal site, rather than individual estuaries throughout the Gulf. Similarly, the U.S. western coastline may act as

a singular nursery where gene-flow among individual estuaries occurs over time. An alternative hypothesis to explain the lack of genetic variation within the northwestern Atlantic could be the result of a recent expansion in response to historical coastal environmental changes during Pleistocene. To investigate this possibility, we ran a secondary analysis that included published data representing Caribbean, Eastern Pacific, and Western Pacific populations.

Northwestern Atlantic versus Caribbean versus Pacific

Similarly, low levels of haplotype diversity (0.5208 ± 0.0909) and nucleotide diversity (0.00156 ± 0.001332) were observed for the secondary dataset (Table 2-5). The inclusion of reference sequences defined an additional five haplotypes (M = 26) and an additional nine polymorphic sites (S = 23), despite the shorter fragment length used. A surprisingly high 86% of the total variation was explained by genetic differentiation between groupings (GoM; NWA; Caribbean; E-Pacific; W-Pacific), which may be largely due to the inclusion of the western Pacific sample, which indicates the biological improbability that *C. leucas* embarks on long trans-Pacific journeys, with open oceanic waters acting as an effective barrier to gene flow. An interesting finding, however, was that the number of mutational events between the western Pacific and GoM matched the number of mutations between the GoM and eastern Pacific haplotypes, and also the GoM to the Caribbean clade haplotypes (Figure 2-5). The evolution of these haplotypes by geographic location can be visualized in two separate lineages on the MSN. The Caribbean lineage (H23; H24; H25) can be seen diverging from the main clade by four

mutational events and another Caribbean lineage can be seen diverging from the main clade by four mutational events (H26). The divergence of this Caribbean clade (Figure 2-6) is particularly interesting, suggesting that the Caribbean could have a genetically unique population, or could potentially group with the southwestern Atlantic population (Karl et al. 2011). Likewise, the divergence of the eastern Pacific lineage, though represented by only two specimens that share the same haplotype, branch from within the GoM phylogroup, suggesting common ancestry. The Isthmus of Panama in a traditionally recognized biogeographic barrier that is known to have previously affected population connectivity between the Pacific and Caribbean after its formation approximately 3.2 mya. The observed relationship between the eastern Pacific samples and Atlantic samples may be reminiscent of gene flow via the Caribbean before the emergence of the Isthmus of Panama (O'Dea et al. 2016). In agreement with Pirog et al. (2019), our ME and ML trees both have moderate support (bs > 56) for separate clades between the northwestern Atlantic and western Pacific (Figure 2-6). Additional genetic subdivision has also been described between the northwestern Atlantic and southwestern Atlantic (Karl et al. 2011).

Historical demography estimates were calculated for both the GoM and NWA, but not the Caribbean due to the small sample size. Both Tajima's D and R2 statistics suggest a constant population size, and lack support for recent expansion (Table 2-7). The time since divergence (τ) of the NWA appears to be twice as large as the GoM, and may indicate a younger age of colonization of this basin compared to the Atlantic. Similarly, the NWA estimate for female effective population size (194.5k) was much

higher than the estimate for the GoM (105.5k). This trend follows the theory of neutral sequence evolution, that genetic diversity will increase with effective population size, while the effects of genetic drift decrease. Given the extremely low levels of haplotype and nucleotide diversity observed within the GoM, it is not surprising that the Atlantic population had higher effective population size estimates.

The results of this study indicate phylogeographic population structuring of *C. leucas* between the Caribbean (Nicaragua) and its northern counterparts, as well as a cryptic barrier of gene flow between the GoM and NWA at the southernmost point of Florida, that acts as a point of mixing between the two populations. The degree to which the Florida Keys act as a zone of increased genetic diversity remains unknown and should be considered regarding conservation of the species. While the Florida Keys acts as a mixing zone between the GoM and NWA, the Caribbean (Nicaragua) population is surprising genetically distinct, as shown by the divergent clade in the phylogenetic tree, suggesting there is no gene flow between Nicaragua and the GoM or NWA. This unique genetic signal may be indicative of a potential freshwater population in Nicaragua, or the Nicaraguan individuals may be mixing with the SW Atlantic population. Larger samples from that region are needed to determine the extent of connectivity of the Caribbean with the GoM or NWA, but also to determine whether the origin of the Bull Shark with Nicaragua could have any association with a relict population that was isolated form the rest of the Atlantic at some point in the past. As such, the estuarine habitats of this region, as well as from each genetically distinct region are critical to the stability and survival of each respective population given the philopatric behavior of females towards

their natal site. The Caribbean (Nicaragua), Gulf of Mexico, and northwestern Atlantic appear to be regions with distinct genetic signatures and should be managed accordingly to best maintain genetic variability of the species.

Relation to Previous Studies

The use of mtDNA to determine population connectivity for stock management of fishes has become relatively common with the increased affordability of genetic analyses. Freshwater fishes typically display stronger geographic population differentiation compared to marine fishes resulting from physiological and physical barriers to gene flow (Bermingham and Avise 1986). Dispersal in the marine realm adds a layer of complexity, as marine organisms exhibit varying degrees of dispersal and population connectivity, often caused by cryptic barriers, or behavioral characteristics (i.e., reproductive philopatry) that limit gene flow. While the dispersal potential for large pelagic species may seem large, the observed dispersal is often limited (Palumbi 1994). For example, both the Shortfin Mako and White Shark show some genetic differentiation across ocean basins, but relatively little structure within oceans (Heist et al. 1996b, Pardini et al. 2001). In contrast, coastal marine species tend to show higher levels of population structuring, potentially resulting from near-shore or estuarine habitat use (Bowen and Avise 1990). Such population structuring has been described between the GoM and NWA for several coastal species, including American oyster (Reeb and Avise 1990), Black Sea Bass, Menhaden, and Sturgeon (Bowen and Avise 1990); However,

many studies have also reported a lack of differentiation between the two regions as well (Buonaccorsi et al. 2001, Pérez-Portela et al. 2018).

Previous elasmobranch studies have identified structuring between the Gulf of Mexico and Atlantic for Blacktip Shark (Keeney et al. 2003), Blacknose Shark (Portnoy et al. 2014), and Bonnethead Shark (Portnoy et al. 2015); However, a lack of differentiation between the Gulf of Mexico and northwestern Atlantic has been reported for Sharpnose Shark (Heist et al. 1996a) and Sandbar Shark (Heist et al. 1995, Portnoy et al. 2010). Additional structuring within the GoM has been described in Blacktip Shark (Hueter et al. 2005) and Blacknose Shark (Portnoy et al. 2014).

This study describes population subdivision between the Gulf of Mexico, the NW Atlantic, the Caribbean Sea (Nicaragua), the eastern Pacific and the western Pacific. The differing degrees to which each species exhibit population structuring highlights the importance for understanding population connectivity and gene flow for each species. As a species divides in to multiple reproductively distinct populations, evolutionary forces begin to act independently within each region. Subpopulations that differentiate genetically may hold unique genetic variation that contains important long-term conservation value for that species. Conservation efforts that incorporate these distinct genetic groupings are necessary for implementing a comprehensive fisheries and habitat management plans.

Conclusion

The Bull Shark is a large coastal species with widespread geographic distribution that utilizes estuarine habitat as nurseries to increase success of juveniles. This study demonstrates the degree to which females display regional philopatric behavior towards their natal sites, by characterizing the Control Region of the mtDNA genome. We described strong phylogeographic genetic structuring between the Gulf of Mexico, Atlantic coastline of the US, and the Caribbean. Overall haplotype diversity observed was very low, though the Gulf of Mexico was significantly lower than both the Florida Keys and northwestern Atlantic. While south Florida acted as a cryptic barrier between the Gulf of Mexico and northwestern Atlantic, there also appeared to be unique mixing at the southernmost point of the Florida Keys, creating a region with higher haplotypic and nucleotypic diversity. Tree phylogeny gave strong support for separating the western Pacific populations into a separate clade, and surprisingly another divergent clade that groups Caribbean lineages from Nicaragua's San Juan River. Samples from the eastern Pacific clustered within the Gulf of Mexico and northwestern Atlantic clade, suggesting common ancestry that was likely the result of ancestral gene flow prior to the emergence of the Isthmus of Panama.

CHAPTER III

GENOMIC DIVERSITY COMPARISONS OF A LARGE COASTAL SHARK, *CARCHARHINUS LEUCAS*, IN THE NORTHWESTERN ATLANTIC

Introduction

Background and Biological Significance

A common misconception regarding large pelagic species, such as the Bull Shark (*Carcharhinus leucas*), is the assumption of no population structuring due to the absence of physical barriers or lack of physiological limitations for long migrations in the marine realm. While the potential to disperse freely may exist, many species exhibit varying degrees of structuring for a variety of different reasons (Pardini et al. 2001, Dimens et al. 2019). Understanding the pattern and degree of dispersal is essential to effectively protect critical habitat and to avoid localized overexploitation (Hellberg et al. 2002, Hueter et al. 2005). Sex biased dispersal has been studied and observed across numerous taxa (Prugnolle and De Meeus 2002), and is characterized by individuals of one sex displaying philopatry, while the members of the other sex disperse to other populations and mediate gene flow. Either sex can display philopatric behavior, but in many elasmobranch species the female returns to its natal site to reproduce. Female philopatry has been documented in many shark species, including Lemon Shark (Feldheim et al. 2002, Schultz et al. 2008), Blacktip Shark (Heupel and Hueter 2001, Keeney et al. 2003), White Shark (Pardini et al. 2001), Sandbar Shark, (Portnoy et al. 2010),

Bonnethead Shark (Portnoy et al. 2015, Díaz-Jaimes et al. 2020), and Bull Shark (Karl et al. 2011, Tillett et al. 2012, Laurrabaquio-A et al. 2019, Pirog et al. 2019).

Conventional tagging and tracking methods have been used to examine sex biased dispersal, but tag loss may be problematic, and it may not be always possible to ascertain the extent by which a tagged individual was reproductively successful (Feldheim et al. 2002). The use of genetic markers circumvents these limitations, and may potentially provide greater insight towards generalized (i.e., population-wide) spatial movements patterns and actual gene flow among the populations of a species. Mitochondrial (mt) markers and nuclear (n) markers differ in their mode of inheritance and this allows for comparisons that may reveal sex-biased dispersal patterns of behavior. The matrilineal mode of inheritance of mtDNA is indicative of female dispersal, whereas nuclear markers represent the genetic contribution of both parents; therefore, if genetic subdivision is supported by mtDNA but not by nDNA, the difference may be indicative of male mediated dispersal (Portnoy et al. 2015, Momigliano et al. 2017, Pazmiño et al. 2017).

To date, genetic analyses of *C. leucas* population structure have employed mtDNA and microsatellite loci (i.e., nuclear DNA), and significant differentiation has been reported with mtDNA but not with microsatellites (Karl et al. 2011, Tillett et al. 2012, Laurrabaquio-A et al. 2019, Pirog et al. 2019). This lack of congruences has been interpreted to be caused by female philopatric behavior. Although microsatellites are widely used to assess population connectivity of many vertebrates, there are many challenges in analysis and interpretation of microsatellite data for population genetic

studies (Estoup et al. 2002, Putman and Carbone 2014). As an alternative, the use of single nucleotide polymorphisms (SNPs) is becoming increasingly popular due to the many advantages within population level studies (Vignal et al. 2002, Brumfield et al. 2003, Morin et al. 2004, Schlötterer 2004, Haasl and Payseur 2011). SNPs are characterized through massive parallel sequencing technologies and can provide a much greater number of informative markers compared to previous methods employed to characterize nDNA variation, such as exon-primed-intron-crossings (EPICs), and microsatellites. Though it is an emerging technique, the analysis of SNP data has already proven useful for identifying sex biased dispersal in coastal shark species (Portnoy et al. 2015, Díaz-Jaimes et al. 2020).

This study seeks to employ next-generation sequencing to examine SNP data, together with the previously characterized mtDNA Control Region from Chapter II, to determine whether the previously reported lack of differentiation with microsatellites is due to male mediated gene flow.

Mitochondrial DNA Analyses Results

In Chapter II, population structure throughout the northwestern Atlantic was determined by characterizing mtDNA Control Region (CR) sequences. Because of its maternal inheritance, all the structure discovered using CR data was attributed to female patterns of gene flow. Significant structuring was described between the Gulf of Mexico (GoM) and northwestern Atlantic (NWA) US coast, with the Florida Keys partitioning separately as a mixing zone between the two populations (Table 2-4). While very low

levels of haplotype diversity were reported across all samples (0.6234 ± 0.01034), the GoM showed significantly lower haplotype diversity than the NWA. No structuring was found within the GoM, with most individuals represented by a single haplotype (> 81%, Figure 2-3). In here, we utilize SNP data obtained with double-digest restriction-site associated DNA (ddRAD) sequencing (Peterson et al. 2012) to generate a random representation of genomic variation throughout the genome to test the hypothesis of no-differentiation among samples from the Gulf of Mexico and the NW Atlantic.

Methods

Sample collection

Tissue samples, consisting of fin clips or biopsy punches were obtained from collaborative studies or surveys for scientific purposes throughout the Gulf of Mexico (GoM), and the northernmost extent of the range for *C. leucas* on the northwestern Atlantic coastline. In the GoM, samples came from Campeche, Mexico (n = 16), Texas (n = 16), Big Bend Region in Florida (n = 7), near Everglades National Park in Florida (n = 8), and Pamlico Sound near North Carolina (n = 10). In total, 57 samples were collected and immediately preserved in 99% ethanol and stored at room temperature, or frozen at -20°C, until assayed.

Double-Digest Restriction-Site Associated DNA (ddRAD) Sequencing

High yields of high molecular weight DNA were obtained using the Zymo *Quick*-DNA Universal Kit following the manufacturer's protocols for tissue extractions (Zymo Research, Irvine, CA, U.S.A.). The quality of DNA isolate was visualized by running 4 µL in a 1% Tris-acetate (TA) agarose gel pre-stained with ethidium bromide (EtBr). The concentration and nucleic acid purity of DNA isolate was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA), followed by quality and quantity checks using a Qubit Fluorometer (Thermo Scientific, Waltham, MA). Samples (n = 57) that yielded the best DNA isolation concentrations (> 50 ng/uL) and molecular weight (> 10,000 bp) were selected for ddRAD sequencing. These samples represented five localities throughout the northwestern Atlantic (NWA; Table 3-1). Samples were sent to the Texas A&M AgriLife Genomics and Bioinformatics facility (Texas A&M University, College Station, TX) for library preparation and paired-end ddRAD sequencing. Studies on the bonnethead shark (Portnoy et al. 2015) and blacknose shark (Dimens et al. 2019) have found success using two 6-base cutter restriction enzymes *Eco*RI and *Sph*I for ddRAD sequencing. Our initial attempts at sequencing followed similar protocols; however, we found greater cutting success substituting one of the 6-base cutting enzymes for a 4-base cutting enzyme that cut at the same site. Therefore, library preparation and paired-end ddRAD sequencing was performed using restriction enzymes *Sph*I and *Mlu*CI, which targets a motif imbedded within the restriction site targeted by *Eco*RI, on the NovaSeq 6000 sequencing system. Raw Illumina reads were demultiplexed using the bcl2fastq Conversion Software, and

had adapters and barcodes removed using cutadapt 1.8 (Martin 2011) before data was released for investigation.

Bioinformatic Analyses

Raw paired-end reads were processed using process_radtags perl script in STACKS v2.3 (Catchen et al. 2013) to filter low quality sequences (Phred > 30). Variant sites were called following parameter determination method described in Paris et al. (2017) using the denovo_map pipeline in STACKS. Assembly within STACKS required a minimum stack depth of 5, distance allowed between loci ≤ 3, and distance allowed between stacks to be ≤ 3. POPULATIONS within STACKS was then used to filter variant sites, allowing 20% missing data per locus across all individuals (-r 0.80). A minimum minor allele frequency of 10% (min_maf 0.10) was set to reduce type I errors by filtering out genotyping errors and uninformative loci (Cupples et al. 2007). To minimize the effects of linkage disequilibrium, only a single SNP per locus was used to determine population structure. Molecular indices for each sampling locality, including mean nucleotide diversity (π), mean observed heterozygosity (H_o), number of loci deviating from Hardy-Weinberg equilibrium (HWE), and the mean inbreeding coefficient (F_{IS}) of individuals (Hartl et al. 1997) were calculated using the POPULATIONS module for these retained biallelic loci.

The number of underlying populations was determined using Evanno log-likelihood metrics and visualized using the software STRUCTURE v0.6.94 (Pritchard et al. 2003, Evanno et al. 2005, Earl 2012). STRUCTURE was chosen due to the

algorithms capability to assign individuals to populations, while also providing testing for the presence of structure. The algorism also has a helpful extension to incorporate geographic labels for each sample under the assumption that each individual likely originated from the corresponding geographic sampling region, but allows a small probability that the individual is from another locality or had an ancestor that migrated from a different locality. The ADMIXTURE model was used due to its flexibility to handling mixed ancestry. STRUCTURE analysis ran 20 independent runs of the Gibbs sampler for each value of *K* (number of populations) between 1 and 4. Results are based on runs of 10^4 iterations, following a burn-in of 15,000 iterations.

We ran a secondary analysis to determine if population structure was being driven by outlier SNPs. PGDSpider v1.0.1.4 (Lischer and Excoffier 2012) was used to exclude loci with only missing data and non-polymorphic SNPs, then convert the VCF file to a BayeScan file in order to filter our variant sites to determine if outlier were driving the population structure. BayeScan v2.1 (Foll and Gaggiotti 2008) was used to identify outliers using default parameters and a false discovery rate (FDR) of 0.05. Outliers were then extracted and samples with high levels of missing data (< 10 loci / sample) were removed. POPULATIONS was used within STACKS to filter variant sites using same parameters described above (-r 0.80; min_maf 0.10).

Results

ddRAD Sequence Data

A total 289,139,348 paired-end ddRAD sequences were obtained for 57 specimens from five sampling locations within the Gulf of Mexico and NW Atlantic. After filtering for low quality reads, an average of 5,072,407 million reads per individual was retained, with only about 213.51 reads lost, on average, per sample (Figure 3-1). Of the 451,094 loci identified, 1% failed to fit to a contig (Avg size = 267.9bp) and were removed. About 96% of the paired-end reads of the remaining 446,459 loci aligned successfully. Filtering first removed 376,594 loci, leaving 69,865 loci composed of 24,698,410 nucleotide sites. The pipeline then filtered the nucleotide sites, retaining 18,174 biallelic variant sites (SNP markers) to assess population connectivity.

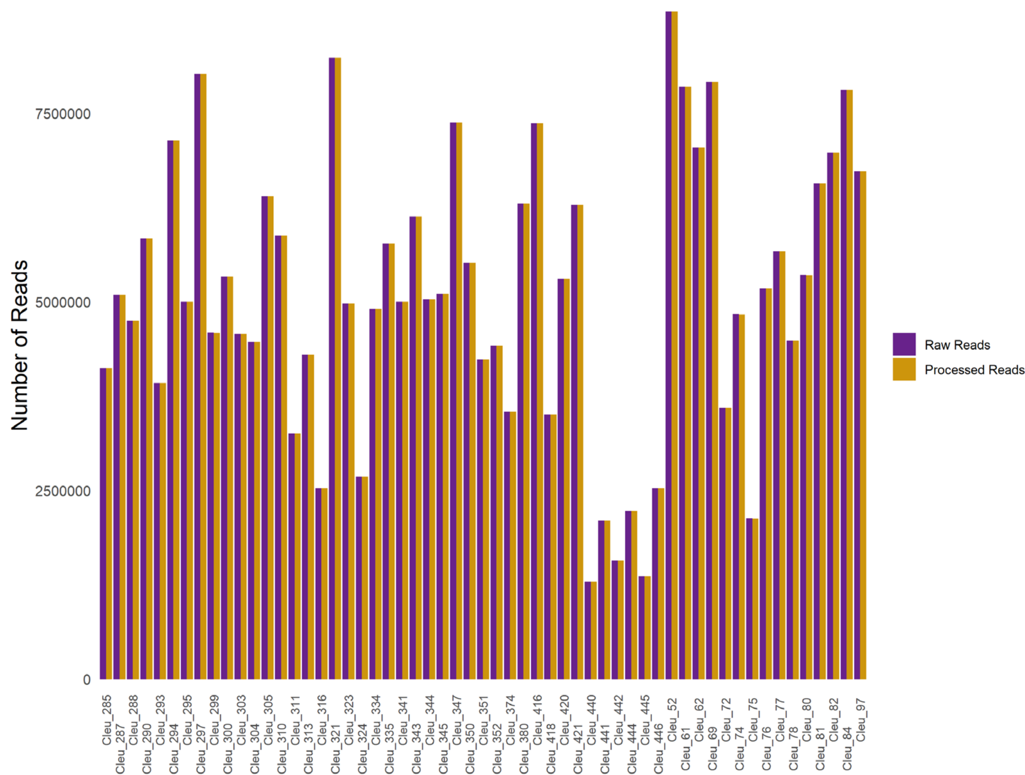

Figure 3-1. Plot visualizing the number of ddRad sequences obtained per individual before and after quality filtering (Raw Sequence Reads vs Processed Reads).

The reported molecular indices for SNPs were calculated using only the variable biallelic sites in each population (Table 3-1). Levels of observed heterozygosity for variant positions ranged from 0.362 – 0.403, with the lowest values found in Pamlico Sound (North Carolina) and the highest values were found in Texas. The number of SNPs not in Hardy-Weinberg Equilibrium (HWE; $p < 0.05$) varied between populations, Texas having the largest number (564 SNPs) and North Carolina the lowest (154 SNPs). The inbreeding coefficient was negative in all locations, but not significantly different from zero. To account for the potential selection effects due to the inclusion of non-

neutral loci, outliers were extracted to determine if the observed structure could be an artifact of selection effects; However, no subdivision observed using only the outlier SNPs subset. This lack of structure suggests the observed population differentiation was not due to selection.

Location	***n***	**HWE**	**H_O(SE)**	**π (SE)**	**F_{IS}(SE)**
Campeche	16	544	0.374 (0.002)	0.3571 (0.0012)	-0.0336 (0.0103)
Texas	16	564	0.403 (0.002)	0.3609 (0.0011)	-0.0950 (0.0101)
NW FL	7	205	0.395 (0.002)	0.3695 (0.0014)	-0.0464 (0.0045)
SW FL	8	290	0.394 (0.002)	0.3642 (0.0014)	-0.0592 (0.0047)
North Carolina	10	154	0.362 (0.003)	0.3481 (0.0020)	-0.0249 (0.0109)

Table 3-1. Molecular indices for all variant positions (SNP markers) within each locality. *n*, Number of samples; HWE, Number of variable sites significantly outside Hardy-Weinberg equilibrium (<0.05); H_O, Mean Observed Heterozygosity; π, Mean nucleotide diversity; F_{IS}, Inbreeding Coefficient; SE, Standard Error.

The Evanno test was used to determine K2 as the most likely fit for population structure given ΔK value. The ADMIXTURE model indicated strong structuring between sampling localities (Figure 3-2). Most samples from Campeche, Mexico were fully assigned in to one cluster (1, Figure 3-2), along with 60% of the samples from North Carolina indicating majority assignment to the same cluster (5). The second discrete cluster contained majority assignment of individuals from Texas, NWFL, and SWFL. While the southernmost tip of Florida appears to limit dispersal between the northern GoM cluster and the Campeche/Atlantic cluster, gene flow between these two

may occur as indicated by the presence of four individuals from North Carolina that belong to the northern GoM cluster and one individual from Campeche fully assigned to the northern GoM cluster.

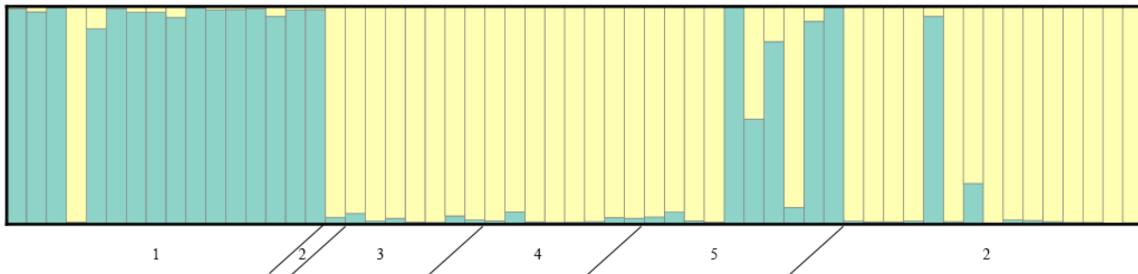

Figure 3-2. STRUCTURE K2 plot derived from SNP data depicting the probabilty of assigning each individual (i.e., each column) to the one of two clusters (yellow or blue). Numbers correspond to sampling localities: 1, Campeche; 2, Texas; 3, NW FL; 4, SW FL; 5, North Carolina.

Discussion

Genetic structure of *C. leucas* has previously been described throughout the northern Gulf of Mexico and the NW Atlantic utilizing mtDNA and microsatellites (Karl et al. 2011, Laurrabaquio-A et al. 2019). While the use of microsatellites is common for determining population differentiation, these markers are not able to yield the same level of full genome coverage as SNP data, and may not reflect genome-wide diversity of natural populations (Ouborg et al. 2010). The use of SNPs in conjunction with mtDNA

provides new insights into distinct structuring of both female and male Bull Shark throughout the NW Atlantic.

STRUCTURE analyses of SNP data using 18,174 variant sites identified discrete populations between the southern GoM (Campeche) and the rest of the GoM. Interestingly, 50% of the individuals sampled near Pamlico Sound (NC) were assigned within the same grouping as the southern GoM individuals. The near complete assignment of each individual within the STRUCTURE output (Figure 3-2) suggests the southernmost tip of Florida acts as a cryptic barrier for gene flow when characterizing nDNA, similar to the pattern obtained with mtDNA CR (Figure 2-3). While population division occurred at the same general location near the southern tip of Florida, the SNP data displayed different patterns of gene flow between the southern GoM and the NWA. MtDNA structure described the entire GoM as a singular population, while SNP data identified the southern Gulf as genetically distinct from the rest of the GoM, suggesting male-biased dispersal is driving the observed differences in gene flow between the southern GoM and NWA.

Interestingly, the nucleotide diversity trends between sampling localities differed between mtDNA and nDNA. MtDNA found the lowest to highest nucleotide diversity as follows, NWFL, SWFL, Texas, North Carolina, and Campeche (Table 2-1); However, the SNP data suggests an inversely proportional relationship with the lowest to high nucleotide diversity as follows, North Carolina, Campeche, Texas, SWFL, and NWFL (Table 3-1). While the nucleotide diversity values for mtDNA and nDNA may not be

directly comparable due to different sampling schemes, the overall trends observed between sampling locations remain informative.

While this study is the first to include representative samples from both the southern GoM and North Carolina to examine population structure of *C. leucas*, our findings contrast with the lack of structure described previously between the northern GoM and NWA using nDNA data (Karl et al. 2011, Laurrabaquio-A et al. 2019). Microsatellite data identified a weak differentiation between the GoM and Brazilian populations, though STRUCTURE analyses were not significant (Karl et al. 2011). Not surprisingly, significant structuring of microsatellite data was described between the GoM and western Indian Ocean, although no structuring was found between the Indian Ocean and the West Pacific (Pirog et al. 2019). The long migration and mixing inferred between western Indian Ocean and West Pacific suggest the observed structuring between the southern GoM and NWA, described in this study, may not due to a physiological barrier limiting gene flow in Bull Shark, but the result of a behavioral preference, instead.

The observed structuring between the northern GoM and NWA (i.e., North Carolina) based on nuclear markers is consistent with other shark species, including Bonnethead Shark (Portnoy et al. 2015, Díaz-Jaimes et al. 2020), and Blacknose Shark (Dimens et al. 2019); However, the observed clustering of the southern GoM with the NWA has not yet been described in other shark species. The movement of *C. leucas* along the Yucatan Peninsula is relatively unknown, though other pelagic species have been tagged and observed performing similar migrations using the Yucatan Channel and

Straits of Florida (Rooker et al. 2019). While our sampling distribution along the Atlantic coast was not exhaustive for the SNP analyses, each individual had a high assignment percentage indicating two populations likely occur. More extensive sampling of the Atlantic coastline to assess genetic assignment, in congruency with tracking data could corroborate the unique pattern of gene flow we have described in this study.

Conclusion

The economic and ecological importance of *C. leucas* necessitates comprehensive management of the species for long term sustainability. The genetic differentiation observed between mtDNA and nDNA suggest a pattern of non-random mating for both sexes throughout the Gulf of Mexico and northwestern Atlantic. Female patterns of gene flow are restricted between the GoM and NWA, with some mixing at the Florida Keys. Male patterns of dispersal are restricted within the northern GoM, with the southern GoM mixing with the NWA. The distinct subdivision of each sex creates uniquely restricted gene flow that should be considered and implemented in future conservation efforts to best maintain viability of the species, though there are additional international management implications given the gene flow between Mexico and the United States.

CHAPTER IV

GENERAL CONCLUSIONS AND IMPLICATIONS

This thesis highlighted the importance of characterizing gene flow and connectivity of a species utilizing markers that differ in mode of inheritance to independently assess the patterns of dispersal of males and females. In Chapter II, significant structuring was described with the maternally inherited mitochondrial DNA (mtDNA) Control Region (CR) used to assess female patterns of dispersal. In Chapter III, significant structuring was described using biparentally inherited single nucleotide polymorphisms (SNPs) obtained from double digest restriction site associated DNA (ddRAD). Observed differences in structure between the mtDNA CR and nuclear SNPs can be attributed to male-biased dispersal.

Population Structure of Maternally Inherited DNA

Phylogenetic analyses of 733 base pairs (bp) of the mtDNA CR revealed regional subpopulations, no isolation by distance, and low haplotype diversity across all samples. Significant population division was found between the Gulf of Mexico (GoM) and northwestern Atlantic (NWA) US coast. Individuals sampled from the southernmost point of Florida (Keys) suggests that this region acts not only as a cryptic barrier of dispersal between the GoM and NWA, but also as an area where the two regions mix. This was shown by the significantly higher levels of haplotype diversity within the Keys

in comparison to both the GoM and NWA. Haplotype diversity across all samples was low, though the GoM had significantly lower haplotype diversity, with the majority of individuals (~68%) expressing the same haplotype.

A secondary phylogenetic analysis of trimmed mtDNA CR (463 bp) including reference sequences from the Caribbean, eastern Pacific, and western Pacific, again revealed strong regional subdivision, no isolation by distance, and low haplotypic diversity. Similar patterns of divergence between the GoM and NWA were observed with the trimmed dataset, though the Keys were grouped within the NWA localities. Interestingly, samples from the Caribbean (Nicaragua) diverge as a separate strongly supported clade, indicative of the absence of female dispersal between the Caribbean and northern Atlantic. The western Pacific samples diverge significantly from all other localities, giving evidence to support historical gene flow between the eastern Pacific and Caribbean subpopulations. This distinct pattern of historical demography is likely attributed to connectivity before the emergence of the Isthmus of Panama (O'Dea et al. 2016). There was no statistical evidence of recent bottlenecks or expansion, suggesting populations in the GoM and NWA are large and relatively stable. Conservative estimates for female effective population size (Ne) for both the GoM and NWA were 105,000 and 194,500, respectively.

Population Structure of Biparentally Inherited SNP Markers

Genomic variation was characterized across the entire genome using 18,174 SNPs, identifying discrete populations resulting from male-biased dispersal. The Florida Keys acted as an effective cryptic barrier for gene flow between the northern GoM and NWA; However, individuals from Campeche, Mexico in the southern GoM were assigned with most (50%) of the samples from North Carolina. The observed structuring of male *C. leucas* connecting the southern GoM and NWA has not yet been described, and relatively little is known about the movement of Bull Shark along the Yucatan Peninsula. As there are no physiological limitations to prevent dispersal between the northern GoM and southern GoM, as shown by mtDNA, we hypothesize that the observed partitioning of regions for male dispersal is behaviorally driven. Similar migratory patterns using the Yucatan Channel and Straits of Florida have been observed between the southern GoM and NWA when tagging large pelagic species (Rooker et al. 2019)

Management Implications

The Bull Shark holds high economic value for the artisanal fisheries in many parts of the world, as well as ecological importance given its high trophic position in the food web. The genetic heterogeneity detected in this study using mtDNA and nDNA suggest independent selective mating patterns of both sexes throughout the GoM and

NWA. Female patterns of gene flow are regionally restricted between the NWA, GoM, and Caribbean, while male patterns of gene flow are regionally restricted within the northern GoM, with the southern GoM individuals mixing with the NWA. Given regions are genetically distinct from one another, this knowledge needs to be incorporated in the future regional management plans of this species to properly define stocks, prevent the unintentional overexploitation of subpopulations, and to conserve the unique genetic patterns of variation throughout this species range.

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APPENDIX A

SUPPLEMENTAL MATERIAL FROM CHAPTER II

Table A-1. Sample collection information for *Carcharhinus leucas*. Region (represents the general location where samples were collected), Sample Type, Number of Individuals (*n*), and Sample Source.

Region	Sample Type	*n*	Source
Texas	Tissue	26	Texas A&M University Galveston
Campeche, Mexico	Tissue	26	Universidad Nacional Autónoma de México
	mtDNA CR Sequences	1	Kitamura et al. 1996
Louisiana	Tissue	27	Louisiana Department of Wildlife and Fisheries
GoM Florida	Tissue	33	Florida State University
	Tissue	3	National Oceanic and Atmospheric Administration
NWA Florida	Tissue	19	Florida Atlantic University
	Tissue	3	NOAA
Mid Atlantic	Tissue	15	NOAA
North Carolina	Tissue	13	Smithsonian Environmental Research Center
	Tissue	2	NOAA
Australia	mtDNA CR Sequences	166	GeneBank (Tillet et al. 2012)
	mtDNA CR Sequences	1	Kitamura et al. 1996
China Straight	mtDNA CR Sequences	1	GeneBank (KF646785)
Nicaragua	mtDNA CR Sequences	6	Kitamura et al. 1996
Teacapan, Mexico	mtDNA CR Sequences	2	Kitamura et al. 1996

Table A-2. Haplotype frequencies for 733 bp of mtDNA sequences for *C. leucas* by Sampling location.

Haplotype	*n*	TX	LA	MX	NWFL	SWFL	KEYS	SEFL	MA	NC
CLEU_CR1	78	15	22	17	12	8	2	2	0	0
CLEU_CR2	9	4	2	3	0	0	0	0	0	0
CLEU_CR3	18	6	3	2	2	0	3	2	0	0
CLEU_CR4	1	1	0	0	0	0	0	0	0	0
CLEU_CR5	1	0	0	1	0	0	0	0	0	0
CLEU_CR6	1	0	0	1	0	0	0	0	0	0
CLEU_CR7	3	0	0	2	0	0	0	1	0	0
CLEU_CR8	24	0	0	0	1	4	2	8	1	8
CLEU_CR9	17	0	0	0	0	1	1	6	7	2
CLEU_CR10	1	0	0	0	0	1	0	0	0	0
CLEU_CR11	1	0	0	0	0	1	0	0	0	0
CLEU_CR12	1	0	0	0	0	0	1	0	0	0
CLEU_CR13	1	0	0	0	0	0	0	1	0	0
CLEU_CR14	1	0	0	0	0	0	0	1	0	0
CLEU_CR15	1	0	0	0	0	0	0	0	1	0
CLEU_CR16	1	0	0	0	0	0	0	0	1	0
CLEU_CR17	1	0	0	0	0	0	0	0	1	0
CLEU_CR18	1	0	0	0	0	0	0	0	1	0
CLEU_CR19	1	0	0	0	0	0	0	0	1	0
CLEU_CR20	3	0	0	0	0	0	0	0	0	3
CLEU_CR21	2	0	0	0	0	0	0	0	0	2
Total:	167	26	27	26	15	15	9	21	13	15