Genetic structure of loggerhead turtle (*Caretta caretta*) and green turtle (*Chelonia mydas*) populations nesting in the Northwest Atlantic inferred from mitochondrial DNA

by

Brian Michael Shamblin

(Under the direction of Campbell J. Nairn)

Abstract

Mitochondrial DNA polymorphisms have been widely utilized to assess demographic connectivity of marine turtle rookeries, including those of the southeastern United States of America (USA). This region hosts the largest nesting aggregation of loggerhead turtles in the Atlantic and one of two globally significant nesting assemblages for the species. Determining the stock structure of the nesting aggregation is important for defining demographically independent nesting populations (management units) and providing appropriate baseline data for mixed stock analyses of foraging aggregations and stranded turtles. Previous studies based on a 390 base pair fragment of the mitochondrial control region partitioned the southeastern USA loggerhead turtle nesting aggregation into four management units. I reassessed the population structure of the nesting aggregation using 834 novel samples collected from North Carolina through the Florida panhandle as well as published haplotype frequency data. Pairwise F_{ST} comparisons, exact tests of population differentiation, and analysis of molecular variance (AMOVA) supported the presence of six management units corresponding to beaches from 1) North Carolina through northeastern Florida, 2) central eastern Florida, 3) southern Florida (southeastern and southwestern), 4) Dry Tortugas, Florida, 5) central

western Florida (Sarasota County), and 6) northwest Florida. Despite the increased resolution gained from expanded sampling and larger sample sizes, the relationship of southernmost rookeries on the Gulf and Atlantic coasts of Florida remained unresolved. To address this question and assess the utility of an expanded control region fragment (817 bp) to refine stock structure, I analyzed 2,260 samples representing twelve rookeries of the southeastern USA aggregation as well as the Cay Sal, Bahamas rookery. This analysis supported the six management units suggested by the earlier study and additionally differentiated southeastern and southwestern Florida as distinct management units. The Cay Sal and Dry Tortugas rookeries were not genetically differentiated and were grouped as a single management unit, although it is probable that these distant rookeries are demographically isolated. As previously demonstrated by a published study, use of the larger control region fragment significantly increased population structure detected between western and eastern Atlantic loggerhead turtle rookeries. However, expanded sequences did not significantly improve resolution of structure among rookeries comprising the southeastern USA nesting aggregation in most comparisons, which were dominated by two common control region haplotypes. The single exception was southeastern Florida rookeries compared to all others because of the high percentage of CC-A1.3 relative to the common CC-A1.1. Given the ability of marine turtles to colonize sites far from their natal regions and the slow evolutionary rate of the mitochondrial genome relative to many other vertebrates, haplotype sharing is a common phenomenon among marine turtle rookeries regionally and in some cases across ocean basins. This haplotype sharing can confound detection of demographic independence of rookeries as well as introduce uncertainty into rookery contribution estimates to mixed foraging aggregations. I explored the utility of mitogenomic sequencing to differentiate green turtle lineages nesting at southern Greater Caribbean rookeries and carrying 490 bp control region haplotype CM-A5. Mitogenomic sequencing revealed four variants of CM-A5 and suggested demographic independence of eastern Caribbean rookeries that were not differentiated based on the 490 bp haplotypes: Buck Island (St. Croix), United States Virgin Islands; Aves Island, Venezuela; and Galibi,

Suriname. Mitogenomic sequencing may resolve several cases of haplotype overlap among marine turtle rookeries and thus improve the resolution of stock structure and mixed stock analyses.

INDEX WORDS: loggerhead turtle, *Caretta caretta*, green turtle, *Chelonia mydas*, population structure, mitochondrial DNA, mitogenome

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Chapter 1

Introduction and Literature Review

Marine turtles have inhabited the world's oceans since at least the Cretaceous period (Hirayama 1998; Kear and Lee 2006). There was once more diversity in the group than is evident today, with only two of five families surviving to modern times (Pritchard 1996). Numerous morphological and genetic datasets have placed the two extant families of marine turtles in suborder Cryptodira, the "hidden necked" turtles, though strong support for the precise placement of marine superfamily Chelonioidea within the suborder has been illusive (eg. Shaffer et al. 1997; Parham et al. 2006; Thomson and Shaffer 2010). The most recent phylogenies utilizing several nuclear genes resolved marine turtles as sister taxa to a clade containing the mud, musk, and snapping turtles (Barley et al. 2010).

Marine turtles are represented by seven extant species distributed primarily in tropical and warm temperate waters globally. The leatherback turtle (*Dermochelys coriacea*) is the sole surviving representative of the family Dermochelyidae. Six species of "hard-shelled" turtles comprise the family Cheloniidae: the loggerhead turtle (*Caretta caretta*), hawksbill turtle (*Eretmochelys imbricata*), Kemp's ridley (*Lepidochelys kempii*), olive ridley (*Lepidochelys olivacea*), green turtle (*Chelonia mydas*), and flatback (*Natator depressus*). Recent phylogenetic research utilizing nuclear as well as mitochondrial genes reaffirms the original placement of the flatback turtle in tribe Chelonini with the green turtle (Naro-Maciel et al. 2008). The remaining species of Cheloniidae belong to tribe Carettini (Bowen et al. 1993; Dutton et al. 1996; Naro-Maciel et al. 2008). The flatback turtle has the most restricted distribution of all the species, nesting only in northern Australia and foraging in adjacent waters (Bjorndal and Jackson 1996). The Kemp's ridley also has a restricted range, nesting mainly at Rancho

Nuevo in Tamaulipas, Mexico and foraging primarily in the Gulf of Mexico and western North Atlantic (Bjorndal and Jackson 1996). The remaining species have more cosmopolitan distributions, with nesting sites occurring in both the Atlantic and Indo-Pacific.

Six of the seven marine turtle species are considered vulnerable, endangered, or critically endangered by the World Conservation Union's Marine Turtle Specialist Group, with the flatback turtle listed as data deficient (Seminoff and Shanker 2008). Marine turtles have a long history of exploitation by humans (eg. Frazier 2002; Allen 2007). In several instances, direct exploitation via turtle "fishing" or egg harvesting reached industrial scales that proved unsustainable for local populations. Europeans heavily exploited green turtles for their meat during exploration and settlement of the New World (Parsons 1962). Based on historical counts and harvest records, the current abundance of green turtles in the Caribbean region is estimated to be less than 1% of pre-Columbian numbers, with a few major rookeries harvested out of existence (McClenachan et al. 2006). Harvest of female loggerhead turtles is locally rampant in some nesting colonies. An estimated 25% of females nesting on Boavista, Cape Verde during the 2007 season were slaughtered (Marco et al. 2010), potentially compromising the second largest nesting population in the Atlantic. In addition to direct exploitation, longline fisheries incidentally capture thousands of loggerheads each year. Between 210,000 and 280,000 loggerheads were hooked by pelagic longline fisheries in the Atlantic and Mediterranean basins in the year 2000, and mortality rates were estimated at 17-42% (Lewison et al. 2004). Annual mean loggerhead turtle nest counts on Floridas index nesting beaches declined by approximately 44% from 1998 through 2006 (Witherington et al. 2009), prompting concern that the largest nesting aggregation for the species in the Atlantic may be in decline. Filling gaps in our knowledge of marine turtle life history is essential for effective management and conservation of these imperiled and highly migratory species. Genetic tools have proven indispensable in addressing these data deficiencies.

The earliest tagging studies of green turtles discovered that individual females often return to specific nesting beaches on subsequent nesting events within a season and across

reproductive seasons (Hendrickson 1958; Carr and Hirth 1962). Upon recognition of this pattern, researchers began to speculate about possible mechanisms that may have given rise to the observed female nest site fidelity. Hendrickson (1958) and Owens et al. (1982) proposed what became known as the "social facilitation" hypothesis. This hypothesis states that virgin females follow experienced females from foraging grounds to natal beaches, and that following a favorable nesting experience, the reproductive recruit imprints on that site for future nesting. Carr (1967) proposed an alternative explanation, the "natal homing" hypothesis, that hatchlings somehow imprint on their natal beach and use these imprinted cues to return to this region as reproductive adults. Given the logistic difficulties of marking sufficient numbers of hatchling sea turtles in such a way as to be able to identify the marks more than a decade later, few direct tests of natal homing have been conducted (but see Bell et al. 2005).

The first indirect test of natal homing using genetic tools was conducted for Caribbean green turtles over twenty years ago (Meylan et al. 1990). Tag recoveries had previously demonstrated that females nesting at the colonies of Tortuguero, Costa Rica and Aves Island, Venezuela utilized overlapping foraging habitats along the coast of Nicaragua (Carr et al. 1978; Gremone and Gòmez 1984), permitting the ideal natural experiment for testing the social facilitation versus natal homing models. The mtDNA restriction fragment length polymorphism haplotype frequencies were significantly different for Tortuguero and Aves nesting colonies with haplotype overlap occurring in only a single individual (Meylan et al. 1990), providing the first genetic evidence of female natal philopatry in marine turtles. Since that time, similar studies have been conducted for all of the marine turtle species with cosmopolitan distributions (reviewed in Bowen and Karl 2007). A general consensus of these studies is that structure has been detected among nesting populations at varying spatial scales (10s of km to 1000s of km) depending on the species in question, and that female natal homing drives this structuring. These demographically partitioned nesting population units are often defined as stocks or management units (sensu Moritz 1994).

Once baseline mtDNA data were collected from major rookeries, the stage was set to utilize genetic data in addressing questions of marine turtle behavior beyond female natal philopatry. Studies aimed at estimating population composition of inwater aggregations of turtles have borrowed heavily from fisheries management methodology. Mixed stock analyses (MSA) were initially designed to estimate stock contributions to catches of anadromous fishes while they were at sea where several populations were admixed (Grant et al. 1980). Early MSAs using genetic data were primarily based on a conditional maximum likelihood approach (Pella and Milner 1987). More recently, Bayesian methods were developed that yield more realistic contribution estimates (Pella and Masuda 2001). These models have become increasingly sophisticated with respect to their ability to incorporate relevant ecological data as covariates (eg. relative sizes of source rookeries and location of potential source rookeries in major current systems; Okuyama and Bolker 2005). A new conceptual framework, many-to-many MSA, was developed to evaluate multiple foraging aggregations simultaneously (Bolker et al. 2007). These mixed stock analyses have proven useful for comparisons for estimation of rookery contribution estimates to aggregations of breeding males as well as foraging adults and juveniles.

Do males also exhibit natal philopatry to breeding sites? The most comprehensive test of male natal philopatry was conducted at three reproductive sites for green turtles in Australia. Characterization of mitochondrial DNA haplotypes demonstrated that rookeries of the Gulf of Carpentaria, the northern Great Barrier Reef, and the southern Great Barrier Reef represented three distinct management units (Norman et al. 1994; FitzSimmons et al. 1997b). Tagging data indicated that turtles representing these rookeries occurred on shared foraging sites (Limpus et al. 1992), thus permitting an unbiased test of natal homing by male green turtles. Pairwise haplotype frequency comparisons for the male and female cohorts at each breeding site indicated no significant difference whereas the haplotype profiles were significantly different among sites, providing the first evidence of regional natal philopatry for male marine turtles (FitzSimmons et al. 1997a). A recent mixed stock analysis of male hawksbill

turtles in breeding condition in the vicinity of Mona Island, Puerto Rico also suggested that a large proportion of males could be attributed to the Mona Island rookery (Velez-Zuazo et al. 2008). However, the presence of some haplotypes among the breeding male cohort and their absence among sampled nesting females did invoke straying to non-natal reproductive sites by a portion of the males (Velez-Zuazo et al. 2008). Further studies are required to better characterize the degree and scale of natal philopatry in male marine turtles across species and populations.

Where do hatchlings from a particular rookery go when they leave the nesting beach? All marine species with the exception of the flatback turtle undergo an oceanic post-hatchling stage (Bolten 2003). Loggerhead, green, and leatherback turtles leaving Florida beaches engage in "swim frenzy" behavior, a period of continuous, hyperactive swimming in which they escape shallow near shore waters and enter the major current systems (Wyneken and Salmon 1992). After the hatchlings leave the vicinity of the natal beach, they are often not seen again in coastal waters until they have grown considerably. The mystery of where the turtles go and how they live prompted Dr. Archie Carr to refer to this life history stage as the "lost year" (Carr 1986).

Carr (1986) speculated that loggerhead turtles leaving western Atlantic natal beaches were transported to the eastern Atlantic by the North Atlantic gyre, and a single tag recovery in the Pacific hinted that hatchling loggerhead turtles leaving their natal beaches in Japan may traverse thousands of kilometers of open ocean to the Pacific coast of Mexico (Uchida and Teruya 1991). Genetic studies of oceanic juveniles have since provided robust evidence of transoceanic transport of small loggerhead turtles in the North Atlantic, North Pacific, and South Pacific (Bowen et al. 1995; Bolten et al. 1998; Boyle et al. 2009). Preliminary evidence also suggests that Cape Verde loggerhead post hatchlings may be carried to the Brazilian coast (Reis et al. 2009). Mixed stock analysis of oceanic juvenile loggerheads foraging in the vicinity of the Azores, Madeira, Andalusia, and the Canary Islands suggest that an overwhelming majority can be attributed to nesting populations in the southeastern

United States (Bolten et al. 1998; Monzòn-Argüello et al. 2009). Similarly, western Atlantic loggerhead turtle stocks are well represented among pelagic juveniles foraging in portions of the Mediterranean Sea (Laurent et al. 1998; Casale et al. 2002; Carreras et al. 2006). A somatic growth model for oceanic juvenile loggerheads in the North Atlantic suggests that the oceanic stage may last from 6.5 to 11.5 years (Bjorndal et al. 2000), at which point turtles begin to recruit to neritic foraging habitats in coastal areas of the northwestern Atlantic.

The oceanic stage of green turtles originating from western Atlantic natal beaches is less well characterized than that of loggerheads. Stable isotope analysis of recent neritic recruits indicate that oceanic green turtles spend the first three to five years of their lives foraging in similar habitats as and feeding carnivorously like oceanic-stage loggerheads (Reich et al. 2007). A tag return from the Azores of a head-started green turtle released from Florida demonstrates that at least some traverse the North Atlantic gyre in a manner similar to loggerhead turtles (Witham 1980). The head-starting program also received tag returns from scattered locations "upstream" of their Florida natal beach throughout the entire Greater Caribbean region (Witham 1980), invoking the possibility that these turtles also traversed at least a portion of the Atlantic gyre prior to recruiting to neritic sites in the Caribbean Sea. Mixed stock analysis of small juvenile green turtles foraging around the Cape Verde Islands indicated a significant contribution from the Suriname rookery (Monzòn-Argüello et al. 2010a). This study also found individuals with haplotypes CM-A1 and CM-A3 at low frequency, and these haplotypes are known only from Greater Caribbean rookeries (Encalada et al. 1996; Bjorndal et al. 2005). Thus transoceanic dispersal of small oceanic juvenile loggerhead and green turtles likely proceeds in both directions in the Atlantic basin. However, the smallest green turtles encountered in neritic coastal foraging habitats in the western Atlantic are considerably smaller than the smallest juvenile loggerhead turtles (20 cm straight carapace length versus 50 cm curved carapace length; Bolten et al. 1998; Reich et al. 2007), suggesting an abbreviated oceanic life stage for green turtles relative to loggerheads.

Which rookeries contribute large juveniles to a particular neritic foraging site? This question shifts the perspective away from the natal beaches and onto select foraging aggregations. Without a means of marking or tagging individuals as hatchlings, genetic tools provide a means of assigning proportional contributions of juvenile foraging aggregations to natal rookeries. Oceanic juvenile loggerhead and green turtles typically recruit to coastal neritic foraging habitats as medium to large juveniles (eg. Bolten 2003). Managers of a particular critical foraging habitat need to know from where the turtles utilizing the site are coming, particularly when the site may be negatively impacted by harvest, stochastic storm events, or environmental catastrophes such as oil spills. Tagging studies suggest that large juvenile green turtles likely recruit through multiple developmental habitats prior to choosing an adult foraging site (Moncada et al. 2006; Bjorndal and Bolten 2008; Senko et al. 2010). Numerous mixed stock analyses of neritic juvenile green turtle foraging aggregations have been conducted in the Atlantic basin (Lahanas et al. 1998; Bass and Witzell 2000; Formia 2002; Bagley 2003; Bass et al. 2006; Naro-Maciel et al. 2007; Bjorndal and Bolten 2008; Monzòn-Argüello et al. 2010a). A general consensus of these studies is that at large spatial scales, nesting populations that occur "upstream" within the same major current system as the foraging site contributed the majority of foraging individuals. Foraging grounds along the east coast of Florida were dominated by contributions from rookeries in Costa Rica, Mexico, and Florida (Bass and Witzell 2000; Bagley 2003). Brazilian foraging grounds were dominated by contributions from south Atlantic rookeries, followed by eastern Caribbean rookeries, but also with detectable contributions from Tortuguero (Naro-Maciel et al. 2007). The relative importance of currents, migratory behavior, size of the source rookery, and distance from the source rookery to the foraging site for predicting the composition of mixed stocks has not been resolved. An extensive study of foraging loggerhead turtles along the USA coastline indicated some degree of natal homing by large juveniles in selection of their foraging sites (Bowen et al. 2004), though this structure was much weaker than that inferred

for nesting females at many of the same sites. It is unclear the extent to which juvenile green turtles may also exhibit this broad scale natal homing.

Mixed stock analysis of juvenile foraging aggregations can also highlight existing gaps in knowledge with respect to the genetic characterization of nesting populations. "Orphan" haplotypes, those recovered from foraging individuals but not attributable to any known nesting population, have been reported from several foraging aggregations (eg. Bowen et al. 2004; Carreras et al. 2006; Naro-Maciel et al. 2007). The presence of orphan haplotypes suggests that sampled nesting populations may be incompletely characterized or that unknown rookeries may exist. In most cases, orphan haplotypes comprise only a small percentage of the total foraging individuals analyzed. However, a recent analysis of juvenile hawksbill turtles foraging in the vicinity of the Cape Verde Islands found that over 85% of the individuals carried haplotypes not known from any nesting population, highlighting that a major hawksbill rookery in the region has not been characterized genetically (Monzòn-Argüello et al. 2010b). Conversely, use of the many-to-many mixed stock analysis may also highlight the presence of major undiscovered foraging sites for particular nesting populations (Bolker et al. 2007). The most recent many-to-many mixed stock analysis of Atlantic loggerhead turtles suggests that the primary foraging grounds of Brazilian and Cape Verde juvenile loggerhead turtles have not yet been located (Monzòn-Argüello et al. 2009).

Although natal homing has been established as the general rule for marine turtles, the precise mechanisms involved and the scale of natal homing remain unclear. Natal homing to breeding sites often occurs as part of a complex life history involving ontogenetic or seasonal migrations by individuals that may encompass entire ocean basins, where individuals from distinct breeding populations mix (anadromous salmonids, reviewed in Allendorf and Waples 1995; many cetacean species, reviewed in Hoelzel 1998; marine turtles, reviewed in Bowen and Karl 2007). Lohmann et al. (2008) proposed that both breeding salmon and marine turtles locate natal regions via a biphasic navigation process first involving magnetic cues to direct long distance ocean migration to the general vicinity of the natal area. Salmon then use local

olfactory cues in choosing their target spawning rivers (Wisby and Hasler 1954); however, the local cues driving fine scale nesting beach selection by marine turtles are less well understood (Lohmann et al. 2008). As such, the precise scale of natal philopatry remains unresolved for many marine turtle species, and may vary across nesting populations within species as well as among species. The presence of long stretches of suitable nesting habitat along continental coastlines, as is the case for the southeastern United States, further complicates assessments of population structure for marine turtles. Nonetheless, given that migratory reproductive behavior contributes significantly to patterns of population structure for these species, properly the defining the scale of natal homing behavior is critical to ensuring that demographically discrete populations receive adequate recognition and protection.

A small but growing data set suggests that precise natal homing by female marine turtles occurs, at least in some species and rookeries. One of the most extreme cases of fine scale structure detected using mitochondrial DNA markers involves the Barbados hawksbill turtle rookeries. Rookeries on the leeward and windward sides of the island, separated by approximately 30 km, were significantly different with respect to mitochondrial haplotype frequencies (Browne et al. 2009). An analysis using nuclear DNA markers reported structure among female green turtles nesting within 8 km of beach at Tortuguero, Costa Rica but failed to detect any structure among females nesting along Melbourne Beach, Florida (Peare and Parker 1996). Assignment tests based on five microsatellite loci detected a signal of fine scale natal homing for female green turtles nesting at two of three pocket beaches analyzed on Ascension Island (Lee et al. 2007). Despite these preliminary data indicative of fine scale natal homing, a potential limitation of the use of nuclear markers to infer demographic connectivity among rookeries at intermediate spatial scales (10s to 100s of km) is that sperm, and therefore nuclear alleles, may be exchanged among rookeries even in the presence of strong natal philopatry of both sexes. This has been demonstrated for northern and southern Great Barrier reef green turtle stocks that are undifferentiated with respect to nuclear DNA but exhibit strong partitioning of mitochondrial DNA haplotypes

of both nesting females and breeding males (FitzSimmons et al. 1997a; FitzSimmons et al. 1997b). In this case, genetic exchange between nesting populations was attributable to mating on foraging grounds or along migratory corridors where turtles from both stocks co-occur (FitzSimmons et al. 1997b). In general, nuclear markers suggest higher levels of gene flow among rookeries than do mitochondrial DNA markers in most cases (Karl et al. 1992; FitzSimmons et al. 1997b; Roberts et al. 2004; Bowen et al. 2005; but see Carreras et al. 2007). Given that marine turtle rookeries flourish or perish based on female recruitment, assessments of demographic connectivity among rookeries should be grounded in the use of mitochondrial DNA markers, irrespective of potential migration-mediated or male-mediated nuclear gene flow (Bowen et al. 2005). Only in cases where nuclear markers detect structure at finer spatial scales than mitochondrial markers should they be used to infer population connectivity with respect to female natal philopatry.

Although mitochondrial DNA data have proven essential in defining marine turtle population structure, inferences of demographic connectivity of rookeries based on mtDNA do have some inherent limitations. Marine turtle mitochondrial genomes evolve slowly relative to those of several other vertebrate groups, possibly due to the slow metabolism and long generation times characteristic of the marine turtle species (Avise et al. 1992; Bowen et al. 1993). Combined with the ability of marine turtles to colonize sites far from their natal regions, this reduced evolutionary rate has lead to extensive sharing of mitochondrial control region haplotypes among rookeries for several species across expansive spatial scales. All known green turtle rookeries from the southern Mozambique Channel in the southwest Indian Ocean to the Brazilian rookeries of Atol das Rocas and Trindade Island are dominated by CM-A8 (Bjorndal et al. 2006; Formia et al. 2006; Bourjea et al. 2007). Green turtle rookeries in the southwest Indian Ocean also share haplotypes with rookeries from Malaysia to the Great Barrier Reef to Micronesia, thousands of kilometers away (A1, A2, and C3; Dethmers et al. 2006; Bourjea et al. 2007). In the western Atlantic, haplotype A was recorded from hawksbill turtles rookeries in Puerto Rico, United States Virgin Islands, Barbados, and

Brazil; haplotype F was recovered from hawksbill rookeries in Belize, Puerto Rico, and the United States Virgin Islands (Bass et al. 1996). Haplotype CC-A1 is the dominant haplotype in loggerhead turtle rookeries in the southeastern USA and Cape Verde in the eastern Atlantic (Encalada et al. 1998; Bowen et al. 2004; Monzòn-Argüello et al. 2009); CC-A2 is the dominant haplotype of the Mexican and Cuban loggerhead turtle rookeries, is the second most common haplotype in the southeastern USA, and dominates the haplotype profile of every characterized rookery in the Meditteranean basin (Encalada et al. 1998; Bowen et al. 2004; Carreras et al. 2007; Ruiz U. et al. 2008; Garofalo et al. 2009; Chaieb et al. 2010). Haplotype A was recorded from all Atlantic, Indian, and western Pacific Ocean leatherback turtle rookeries sampled, being absent only in the eastern Pacific stocks (Dutton et al. 1999).

The extensive marker overlap among rookeries can sometimes confound assessment of demographic connectivity or isolation of nesting populations. Does the marker overlap result from a recent shared demographic history and slow evolutionary rate of the marine turtle mitochondrial genome, ongoing exchange of a few females such that genetic divergence is unlikely, or contemporary exchange of sufficient numbers of nesting females to sustain rookeries demographically? Discerning among these scenarios is important for conservation and management purposes on ecological time scales, but lack of resolution of the mtDNA marker may limit the ability of researchers to tease out this information. Despite 1200 kilometers of separation, Aves Island and Suriname green turtle rookeries are comprised almost entirely of females with haplotype CM-A5, making them indistinguishable with respect to control region haplotype frequencies (Encalada et al. 1996). Green turtle rookeries of Bioko, Equatorial Guinea and Ascension Island, 2800 kilometers apart in the South Atlantic, were dominated by CM-A8 and not significantly different with respect to haplotype frequencies (Formia et al. 2006). The haplotype frequencies of green turtle rookeries of the Ngulu and Elato Atolls in Micronesia were not significantly different despite over 900 kilometers of Pacific Ocean expanse between the islands (Dethmers et al. 2006). In the Meditteranean, loggerhead turtle rookeries of Cyprus, Crete, and the Kuriat Islands, Tunisia separated by

500 to 2000 kilometers, share CC-A2 at 100% frequency (Carreras et al. 2007; Chaieb et al. 2010).

Even in cases where it is clear that rookeries are demographically partitioned on the basis of haplotype frequency differentiation, overlap of genetic markers among stocks can confound mixed stock analyses. Despite analytical improvements that have permitted incorporation of ecological covariates as well as the new conceptual framework that allows assessment of multiple foraging aggregations simultaneously, overlap of genetic markers among source populations introduces considerable uncertainty into point estimates of rookery contribution to mixed stocks in some instances (Okuyama and Bolker 2005; Bolker et al. 2007). Credible intervals for point estimates of rookery contributions are often so broad that they encompass a large proportion of total available parameter space because the genetic markers are only weakly informative in several cases (Bolker et al. 2007).

Another limitation of the quality of data from mixed stock analyses relates to the completeness to which haplotype frequencies for potential source rookeries have been assessed. An important assumption of mixed stock analysis is that all potential source populations have been thoroughly characterized. This assumption has only partially been fulfilled for Atlantic green turtle and loggerhead rookeries. The two major Atlantic green turtle rookeries of Tortuguero, Costa Rica and Ascension Island have been thoroughly analyzed with respect to control region haplotype frequencies (Bjorndal et al. 2005; Formia et al. 2007). However, regionally significant rookeries in Florida are still represented by relatively small sample size of the initial studies that were limited by permitting agencies (Bowen et al. 1992; Encalada et al. 1996). Moreover, nesting occurs over several hundreds of km of the Florida coastline(Witherington et al. 2006), allowing the possibility that samples taken from a small fraction of potential nesting habitats may not be representative for the entire nesting aggregation. Though genetic characterization of loggerhead turtle rookeries in the southeastern USA is more advanced than for green turtle rookeries in the region, several questions remain with respect to the relationships among loggerhead turtle rookeries in Florida as well.

Addressing data deficiencies and ambiguities regarding demographic inferences requires more thorough sampling of rookeries and deeper sequencing of the mitochondrial genome. In chapter 2, we examine the genetic structure among southeastern USA loggerhead rookeries using a 390 base pair (bp) fragment of the mitochondrial control region using 847 novel samples as well as published haplotype frequency data from previous studies. In chapter 3, we revisit this question using an expanded mitochondrial control region fragment (817 bp) and significantly increased sample sizes for most of the major Florida rookeries. In chapter 4, we explore the use of mitogenomic sequencing to identify informative variation in the CM-A5 lineage to improve the resolution of population structure among green turtle rookeries in the southern greater Caribbean region. In chapter 5, I synthesize the findings of these studies and outline possible future avenues of research.

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Chapter 2

Genetic structure of the southeastern United States loggerhead turtle nesting aggregation: evidence of additional structure within the peninsular Florida recovery unit[1]

[1]Shamblin BM, Dodd MG, Bagley DA, Ehrhart LM, Tucker AD, Johnson C, Carthy RR, Scarpino RA, McMichael E, Addison DS, Williams KL, Frick MG, Ouellette S, Meylan AB, Godfrey MH, Murphy SR, Nairn CJ. Accepted by Marine Biology. Reprinted here with permission of the publisher, 2/28/2011.

Abstract

The southeastern United States supports one of two large loggerhead turtle (*Caretta caretta*) nesting aggregations worldwide and is therefore critical to global conservation and recovery efforts for the species. Previous studies have established the presence of four demographically distinct nesting populations (management units) corresponding to beaches from 1) North Carolina through northeastern Florida, 2) peninsular Florida, 3) the Dry Tortugas, and 4) northwest Florida. Temporal and geographic genetic structure of the nesting aggregation was examined utilizing partial mitochondrial control region haplotype frequencies from 834 samples collected over the 2002 through 2008 nesting seasons from 19 beaches as well as previously published haplotype data. Most rookeries did not exhibit interannual genetic variation. However, the interannual variation detected did significantly impact the interpretation of spatial genetic structure in northeastern Florida. Based on pairwise F_{ST} comparisons, exact tests of population differentiation, and analysis of molecular variance, the present study upholds the distinctiveness of the four currently recognized management units and further supports recognition of discrete central eastern, southern (southeastern and southwestern), and central western Florida management units. Further subdivision may be warranted, but more intensive genetic sampling is required. Additional tools, such as telemetry and mark-recapture, are required to overcome the limitations of the genetic marker in resolving loggerhead turtle rookery connectivity in the southeastern USA.

Introduction

Defining population boundaries for highly vagile marine species often presents challenges given the lack of apparent barriers to movement across sometimes vast spatial scales. For some such species, natal homing behavior to specific reproductive sites dictates population boundaries. Natal homing to breeding sites often occurs as part of a complex life history involving ontogenetic or seasonal migrations by individuals that may encompass entire ocean

basins, where individuals from distinct breeding populations mix (anadromous salmonids, reviewed in Allendorf and Waples 1995; many cetacean species, reviewed in Hoelzel 1998; marine turtles, reviewed in Bowen and Karl 2007). Lohmann et al. (2008b) proposed that both breeding salmon and marine turtles locate natal regions via a biphasic navigation process first involving magnetic cues to direct long distance ocean migration to the general vicinity of the natal area. Salmon then use local olfactory cues in choosing their target spawning rivers (Wisby and Hasler 1954); however, the local cues driving fine scale nesting beach selection by marine turtles are less well understood (Lohmann et al. 2008b). As such, the precise scale of natal philopatry remains unresolved for many marine turtle species, and may vary across nesting populations within species depending on local biotic and abiotic conditions. The presence of long stretches of suitable nesting habitat along continental coastlines further complicates assessments of population structure for marine turtles. Nonetheless, given that migratory reproductive behavior contributes significantly to patterns of population structure for these species, properly the defining the scale of natal homing behavior is critical to ensuring that demographically discrete populations receive adequate recognition and protection.

Loggerhead sea turtles occur globally in warm temperate and tropical waters, though nesting effort is typically focused on warm temperate beaches (Bolten 2003). Loggerhead turtles nesting in the western North Atlantic have a complex life history marked by extensive developmental migrations and seasonal migrations. Genetic analyses and size frequency data have provided strong evidence that loggerhead turtles originating from western North Atlantic beaches spend their early years as pelagic foragers in the eastern Atlantic (Bolten et al. 1998). Broad-scale natal homing by neritic juveniles is supported by mixed stock analysis of several aggregations foraging along the continental shelf of the eastern United States (Bowen et al. 2004). Upon reaching sexual maturity, females migrate to their natal regions to nest (Bowen et al. 1993; Bowen et al. 1994; Bowen et al. 2005). Defining the spatial scale of

this natal neighborhood is an important consideration for delimiting population boundaries, particularly across continuous nesting habitats.

The southeastern United States of America (USA) loggerhead turtle nesting aggregation is one of two globally significant nesting populations, the other being Masirah and other islands along the coast of Oman in the Arabian Sea (Dodd 1988; Baldwin et al. 2003). Loggerhead turtle nesting densities vary considerably over the southeastern USA coastline; approximately 69% of the loggerhead turtle nesting in Florida takes place on 411 km of the 1300 km of surveyed beaches (Witherington et al. 2009). Annual mean nest numbers on Floridas index nesting beaches declined by approximately 44% from 1998 through 2006 (Witherington et al. 2009), prompting concern that the largest nesting population in the Atlantic may be in decline.

In the USA, management and protection of loggerhead turtles is jointly the responsibility of National Oceanographic and Atmospheric Administrations National Marine Fisheries Service (NMFS) and the United States Fish and Wildlife Service (USFWS). Defining the boundaries of nesting populations for management and conservation purposes is a critical element of the recently updated Recovery Plan for the Northwest Atlantic Population of the Loggerhead Sea Turtle (*Caretta caretta*) (hereafter Recovery Plan, NMFS and USFWS 2008).

Numerous concepts have been proposed to identify and classify intra-specific units for conservation or management purposes, and many of these incorporate genetic data (reviewed in Fraser and Bernatchez 2001). Management units, as defined by Moritz (1994), have formed the basis of characterizing loggerhead turtle population structure in the Atlantic basin (Encalada et al. 1998; Bowen et al. 2005). Management units represent populations connected by such low gene flow that they are functionally independent and are recognized as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci (Moritz 1994). In the case of marine turtle populations, rookeries are demographically distinct entities based on female natal philopatry, irrespective of the level of nuclear gene

flow (Avise 1995; Bowen et al. 2005). Thus, significant divergence of mitochondrial haplotype frequencies between rookeries suggests demographic partitioning, which qualifies each rookery as a distinct management unit. For the purposes of the Recovery Plan, the Atlantic Loggerhead Sea Turtle Recovery Team chose to designate intra-specific conservation units known as recovery units. Recovery units are subunits of the listed species that are geographically or otherwise identifiable and essential to the recovery of the species. Recovery units are individually necessary to conserve genetic robustness, demographic robustness, important life history stages, or some other feature necessary for long-term sustainability of the species (NMFS and USFWS 2008). Genetic data have been used as the basis for recovery unit designations where such data are available (NMFS and USFWS 2008). We will use management unit to describe demographically and genetically distinct nesting populations in the spirit of Moritz (1994) and recovery unit only in the context of agency designations outlined above.

Genetic structure among rookeries comprising the southeastern USA loggerhead turtle nesting aggregation has received considerable attention. Restriction fragment length polymorphism analyses of mitochondrial DNA provided strong support for regional natal homing by loggerhead turtles and established the presence of at least two distinct populations nesting in the USA (Bowen et al. 1993; Bowen et al. 1994). Based on significant differences in frequencies of sequence-defined haplotypes and geographic considerations, Encalada et al. (1998) proposed a minimum of three demographically independent nesting populations in the southeastern USA corresponding to beaches from 1) North Carolina through northeast Florida, 2) central and southern peninsular Florida, and 3) northwest Florida. Pearce (2001) analyzed mitochondrial haplotype frequencies and allele frequencies at five microsatellite loci of the original and additional southeastern USA samples. Mitochondrial control region analysis supported previous management unit groupings and added the Dry Tortugas rookery as a distinct management unit (Pearce 2001). Structure inferred from nuclear markers was much weaker than structure inferred from mitochondrial markers, presumably due to weaker natal philopatry in some males or male-mediated gene flow facilitated by turtles from dif-

ferent rookeries mixing along migration routes or on foraging grounds (Pearce 2001; Bowen et al. 2005). Male-mediated gene flow does not detract from the classification of rookeries as independent populations given the fact that female natal site fidelity defines reproductive population boundaries, irrespective of male behavior (Bowen et al. 2005).

Whereas geographic structure among rookeries has been clearly demonstrated in several marine turtle species using mitochondrial DNA tools (reviewed in Bowen and Karl 2007), it is uncertain whether temporal variation in mitochondrial haplotype frequencies at rookeries may also occur. Undetected temporal variation in haplotype frequencies at rookeries could affect the interpretation of spatial structuring among rookeries as well as the integrity of estimates of rookery contributions to juvenile foraging aggregations. Explicit tests of interannual variation in haplotype frequencies have been conducted at a few marine turtle rookeries, and none have detected any statistically significant temporal structuring. Hatase et al. (2002) did not detect significant haplotype frequency variation between two sampling years at four Japanese loggerhead turtle rookeries. The pooled sample was dominated by a single haplotype (Haplotype B = 89%, Hatase et al. 2002), potentially limiting the power to detect any temporal differences. Tests for intraseasonal and interannual variation in haplotype frequencies among green turtles nesting at Tortuguero, Costa Rica also failed to detect any significant temporal structuring (Bjorndal et al. 2005). However, the authors cautioned that the results should be tempered by the recognition that the tests likely had low statistical power given the high frequency of the common haplotype (CM-A3 $>$ 90%, Bjorndal et al. 2005). Similarly, no significant interannual variation was found at the Mona Island hawksbill turtle rookery sampled in 1993, 2003, 2004, and 2005 (Velez-Zuazo et al. 2008). Whether haplotype frequencies are stable at relatively low-density rookeries is unclear, and temporal variation may have important implications for spatial structuring and management unit designations for the southeastern United States loggerhead turtle nesting aggregation given the wide range of nesting densities at different rookeries.

Despite increased resolution with each previous investigation, questions of management interest remain regarding genetic structure among rookeries along the southeastern USA coast. The Recovery Plan currently recognizes four recovery units nesting in the southeastern United States roughly concordant with previous genetic analyses: 1) the northern recovery unit corresponding to beaches from Virginia through the Georgia/Florida border, 2) the peninsular Florida recovery unit, corresponding to all eastern Florida beaches and those in central and southern western Florida, 3) the Dry Tortugas in the Gulf of Mexico off the southwest coast of Florida, and 4) the northern Gulf of Mexico recovery unit, corresponding to beaches in northwestern Florida through the Texas/Mexico border (NMFS and USFWS 2008). It is uncertain whether these recovery units adequately reflect the level of genetic differentiation present among rookeries within the southeastern USA nesting aggregation given low power to detect frequency differences based on small sample sizes for some rookeries. Given that local threats to females concentrated in the vicinity of their nesting beaches will have pinpoint impact on the corresponding nesting population (Bowen et al. 2005), it is critical to recognize genetic structuring and define management units at appropriate spatial scales. An important unresolved question is determining whether a precise boundary exists between the northern management unit and the remaining Florida rookeries. Encalada et al. (1998) anticipated the boundary would occur between Cape Canaveral and Jacksonville based on an established biogeographic discontinuity and the sharp decline in loggerhead turtle nesting density north of Canaveral. Initial analysis of samples obtained from Volusia County suggested that this nesting population represented a distinct management unit (Francisco et al. 1999). However, pairwise Volusia County and Melbourne population comparisons based on a larger Melbourne sample size were not significantly different, prompting Pearce (2001) to include Volusia County within the South Florida management unit.

We re-assessed population genetic structure among rookeries in the southeastern USA loggerhead turtle nesting aggregation by sequencing of a portion of the mitochondrial control region of 834 samples collected during the 2002-2008 nesting and hatching seasons to: 1) test

for interannual variation in haplotype frequencies at individual rookeries, 2) determine the number of management units comprising the southeastern U.S. nesting aggregation and identify potential boundaries, and 3) compare the recovery unit groupings designated in the Recovery Plan with the structure suggested by haplotype frequency and demographic data.

Methods

Field Methods

Samples from 834 individual loggerhead turtles or nests were collected from 19 different southeastern USA beach locations over the 2002-2008 nesting seasons (Table 2.1). Sample sites were chosen to represent the extent of loggerhead turtle nesting in the USA where nesting densities were sufficient to provide adequate sample sizes (Fig 1 and Fig 2). Sites typically included the highest density nesting beaches within each respective region. Each rookery is represented by either samples obtained directly from nesting females or by nest contents obtained during post emergence nest evaluations. Samples from nesting females were collected from the shoulder region using 6-mm biopsy punches following oviposition and during the nest covering and camouflaging process. Precautions were taken to ensure that each nesting female was represented in each annual dataset only once, either via tagging to prevent duplicate sampling, or by using microsatellite genotyping that would allow recognition of individual turtles (Shamblin et al. 2007, 15 loci, microsatellite data not shown). Nest samples were comprised of tissue from dead hatchlings or hatched eggshells collected during post-emergence nest evaluations, and each nest was represented by a single sample. Sampled clutches were laid June 15 through June 24, 2006; June 15 through June 24, 2007; and June 17 through June 26, 2008. A ten-day sampling window was chosen to maximize sample sizes while minimizing the probability of re-sampling females. The average inter-nesting interval for southeastern USA loggerhead turtles is approximately 14 days, with females rarely re-nesting at fewer than 11 days (reviewed in Dodd 1988). Samples were stored in 95% ethanol prior to DNA extraction.

Laboratory Methods

Genomic DNA was extracted using the DNeasy blood and tissue kit (QIAGEN) following standard protocols. Polymerase chain reaction (PCR) amplifications of a 390 bp portion of the mitochondrial control region were carried out using primers TCR5 and TCR6 (Norman et al. 1994). Universal M13 primer sequences were added to the 5 end of each PCR primer to facilitate sequencing. PCR reactions were carried out in 10 μl volumes containing 10mM Tris, pH 8.4; 50 mM KCl, 1.0 μM of each primer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 unit of *Taq* DNA Polymerase, and approximately 25-75 ng of genomic DNA. PCR cycling parameters were as follows: 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds; and a final extension of 72°C for 10 minutes. PCR products were purified by adding 2 μl of ExoSAP-IT®(USB Corporation) to 7 μl of PCR amplicon and incubated according to manufacturer's instructions. The mtDNA amplicons were sequenced in both directions using ABI BigDye v3.1 (PE Applied Biosystems) and an ABI 3730*xl* DNA Analyzer. Negative controls were included in each batch of PCR amplifications and sequencing reactions to detect contamination.

Data Analysis

Sequences were aligned, edited, and compared to previously described haplotypes using the program Sequencher 4.2 (Gene Codes Corporation). Sequences were assigned haplotype designations after nomenclature published on the Archie Carr Center for Sea Turtle Research (ACCSTR) website (http://accstr.ufl.edu/ccmtdna.html).

Samples producing novel or ambiguous sequences were subjected to a second round of DNA extraction, PCR amplification, and sequencing for verification. Novel haplotypes were deposited with Genbank and ACCSTR. Haplotype frequency data from Encalada et al. (1998), Francisco et al. (1999), Pearce (2001), and Bowen et al. (2005) were included in analyses to test for temporal variation and to fill in the geographic gap at Dry Tortugas for the present study (Tabl33).

Haplotype diversity (h), nucleotide diversity (π), pairwise exact tests of population differentiation, and analysis of molecular variance (AMOVA) were conducted using the software Arlequin version 3.1 (Excoffier et al. 2005). Haplotype diversity was estimated based on Nei (1987). Nucleotide diversity was calculated assuming the model of Tamura and Nei (1993). Significance values for AMOVA were obtained from 10,000 permutations. Exact tests of population differentiation were conducted with 100,000 permutations and 10,000 dememorization steps after the method of Raymond and Rousset (1995). These statistics were used to test for temporal as well as spatial structure. For the purposes of temporal tests, BHI samples were compared with previously collected samples from Bald Head Island, Cape Lookout, Topsail Beach, Camp Lejeune, and Caswell Beach (NC, Encalada et al. 1998); WAS samples were compared with previously collected samples from Little Cumberland and Cumberland islands, Georgia (SGA, Encalada et al. 1998); CSK was compared with samples previously collected more broadly in Sarasota County (SAR, Encalada et al. 1998, Pearce 2001); and SBR was compared with samples previously collected near the Port Everglades inlet (PEV, Pearce 2001). All interannual samples for each site that were not significantly different were pooled for spatial analyses. Because nest samples represent unknown turtles, combining sample sets across more then two consecutive years may have resulted in individual nesting females being represented in the data set more than once.

Following pairwise F_{ST} comparisons and exact tests of population differentiation, all proximal sample sites that were not significantly different were pooled for further analyses. In the case of ambiguous pairwise comparisons, several a priori sample-clustering iterations were performed and examined using pairwise tests and AMOVA. Optimal rookery clusters were chosen by maximizing F_{CT} (genetic variation occurring among management units) and minimizing F_{SC} (genetic variation occurring among sampled rookeries within defined management units) in an AMOVA framework. Significance of the final round of pairwise F_{ST} comparisons and exact tests of population differentiation were adjusted using sequential Bonferroni correction with a table-wide α of 0.05 (Rice 1989).

Results

Haplotype and Nucleotide Diversity

Sequence analysis of newly collected samples identified thirty polymorphic positions, corresponding to 22 transitions and eight indels (Table 2.2). Position 358 contained both an indel and a transition. The variable positions resolved nine haplotypes, eight of which have previously been described from loggerhead turtles nesting in Florida (Bowen et al. 2005). The new haplotype contained an A to G transition at position 119 and has been designated CC-A43 (Genbank accession number EF396287). All 12 haplotypes in the pooled data set belonged to two phylogenetically distinct haplogroups (Fig 3) as previously described by Encalada et al. (1998).

Haplotypes CC-A1 and CC-A2 accounted for approximately 94% of all individuals sampled, but these haplotypes were not randomly distributed (Table 2.3). Haplotype CC-A9, previously described from Quintana Roo, Mexico, and the Dry Tortugas, was detected for the first time on mainland Florida Gulf coast nesting areas. Haplotype CC-A14, previously described from peninsular Florida beaches, was detected among northwest Florida samples. Haplotypes CC-A5, CC-A11, and CC-A13, each represented by single nesting females from Florida nesting beaches in previous analysis (Bowen et al. 2005), were not found in the present study. Haplotype diversity ranged from 0 to 0.615 (Table 2.3), and there was a strong latitudinal component to the haplotype frequency distribution with the northernmost and southernmost beaches exhibiting the lowest haplotype diversity.

Interannual Variation

Annual samples from BHI and CAP were fixed for haplotype CC-A1, and therefore exhibited no temporal variation between sampling years. Comparisons among annual samples at 12 sites using AMOVA suggested differentiation among sample years at the VOL and CSB rookeries (Table 2.4). Pairwise F_{ST} values suggested that the previously collected CSB

sample set (Pearce 2001) was differentiated from the 2002 (F_{ST} = 0.33237, p = 0.02257) and 2004 (F_{ST} = 0.40552, p = 0.04930) samples, but none of the remaining comparisons were significant. Exact tests of population differentiation indicated a significant difference only between the original CSB sample and 2002 (p = 0.02182). Pairwise F_{ST} comparisons among VOL annual samples yielded significant differences for 1998 and 2006 (F_{ST} = 0.11984, p = 0.01673) and 1998 and 2008 comparisons (F_{ST} = 0.08205, p = 0.02297). These annual classes were also significantly different as measured by exact tests of population differentiation (1998 and 2006, p = 0.01445; 1998 and 2008, p = 0.02034). Additionally, an exact test of population differentiation indicated a significant difference between the 2006 and 2007 VOL samples (p = 0.02793). Despite lack of a signal of differentiation with AMOVA at MEL, pairwise F_{ST} comparisons and exact tests of population differentiation indicated a significant difference between the 1996 and 2006 samples (F_{ST} = 0.04768, p = 0.03287; exact test p = 0.01751). None of the remaining pairwise comparisons or exact tests of population differentiation within sites were significant. The pooled WAS and SGA samples were labeled as GA. The pooled SAR and CSK samples were labeled as CSK, and the pooled PEV and SBR samples were designated SBR in spatial analyses.

All undifferentiated temporal samples were pooled for spatial population comparisons. The original CSB sample (Pearce 2001) was treated as a discrete unit (CSB1) with the remaining samples pooled (CSB2). The 2006 and 2008 VOL samples were pooled (VOL1), as were the 1998 and 2007 samples (VOL2). Pooling of MEL annual samples was ambiguous as haplotype frequencies of the oldest sample (Encalada et al. 1998) were not different from those of 1996 or 2006. Because this sample was so small (n = 6) relative to nesting effort (>450 nests/km; NMFS and USFWS 2008) and compared with the other year samples, it was excluded from spatial analysis. The 1996 and 2006 MEL samples were treated as discrete sample units for spatial analysis (MEL1 and MEL2, respectively).

Population Structure

With all sample sites treated discretely including two temporal samples each from VOL, MEL, and CSB, there were 23 sample units. Among the 253 pairwise comparisons, 170 of the pairwise F_{ST} comparisons and 166 of the exact tests of population differentiation were significant without correction for multiple tests (Table 2.5). Most non-significant comparisons were between adjacent sample sites within regions, between sites at similar latitude across the axis of the Florida peninsula, or involved a site with small sample size ($n < 20$). Haplotype frequencies produced a slightly skewed mirror image across the axis of the Florida peninsula with rookeries paired across northwest and northeast Florida, central western and central eastern Florida, and southwestern and southeastern Florida having similar and not significantly different haplotype frequencies, in these respective pairings (Table 2.5, Figure 2). Results from pairwise F_{ST} comparisons and exact tests of population differentiation were generally consistent with seven regional groupings: 1) North Carolina through Georgia, 2) northeastern Florida, 3) central eastern Florida, 4) southeastern and southwestern Florida, 5) Dry Tortugas, 6) central western Florida, and 7) northwestern Florida.

Some proximal inter-regional comparisons produced ambiguous results with all sample sites treated discretely. Haplotype frequencies of CSB1 and SGI from northwest Florida were not significantly different from those of CSK in central western Florida in pairwise F_{ST} comparisons. None of the haplotype frequencies of the small sample units from northwest Florida (SGI, CSB1, and STJ) were significantly different from those of CSK in central western Florida with respect to exact tests of population differentiation. Additionally, frequencies at MID in southeastern Florida were not significantly different from frequencies at DRT in the Gulf of Mexico with an exact test. To address whether these ambiguities were related to small sample sizes, iterations of pairwise comparisons were performed with alternative a priori sample site clustering to determine the most appropriate regional groupings for final comparisons. Comparisons for combined SGI/CSB2 versus CSK and combined SGI/CSK versus CSB2 were both significant, but the former comparison yielded the stronger signal

of differentiation (pairwise F_{ST} = 0.12756, p < 0.00001; exact test p = 0.00063, compared with pairwise F_{ST} = 0.10946, p = 0.00098; exact test p = 0.00664). Combined STJ/CSB2 versus CSK yielded a stronger signal of differentiation (exact test p = 0.00017) than did combined STJ/CSK versus CSB2 (exact test p = 0.02103). Combined CSB1/CSK versus CSB2 yielded a stronger signal of differentiation than did combined CSB1/CSB2 versus CSK (F_{ST} = 0.13670, p = 0.00098; exact test p < 0.00001, compared with F_{ST} = 0.09814, p < 0.00001; exact test p = 0.00735). However, as CSB1 was clearly an outlier relative to more recent sample sets from CSB and other rookeries in the region, CSB1 was pooled with all other northwest Florida samples. The SBR versus combined MID/DRT test was not significantly different (exact test p = 0.15578), whereas combined SBR/MID haplotype frequencies were significantly different from frequencies at DRT (exact test p = 0.00442).

Regional affiliation of the northeast Florida sample units was not clear (Table 2.5). Haplotype frequencies at AML were not different from those of any sample units north of VOL. Haplotype frequencies of SJC, FLG, and VOL1 were significantly different from those of the northern rookeries and CAN, MEL1, and MEL2 to the south. Haplotype frequencies of VOL2 and NSB were significantly different from those of AML and rookeries north, but not from those of SJC or FLG to the north or CAN and MEL2 to the south. Therefore, there were no clear boundaries as haplotype frequencies transitioned clinally. To resolve the most appropriate rookery clustering, several iterations of AMOVA were performed for two cases: 1) recognition of a distinct northeastern Florida management unit and 2) absorption of this region into northern and central eastern Florida management units. Clustering of remaining rookeries was consistent with results from pairwise tests and held constant across all AMOVA iterations: northern sites (NC, CAP, GA); central eastern Florida (CAN, MEL); southern Florida (JUN, FTL, SBR, MID, and KEY); the Dry Tortugas (DRT); central western Florida (CSK); and northwestern Florida: (SGI, CSB, and STJ). A total of 16 rookery-clustering scenarios were considered (Supplemental Table 2.6).

With no northeastern Florida management unit recognized, the optimal clustering was achieved by placing AML, SJC, FLG, and VOL1 within the northern management unit and placing VOL2 and NSB within the central eastern Florida group (scenario NEFL 5, Supplemental Table 2.7). With recognition of a northeastern Florida management unit, the optimal clustering was produced by grouping SJC, FLG, and VOL1 sample into a northeastern Florida management unit while AML was grouped with the northern management unit and VOL2 and NSB were grouped with CAN and MEL (scenario NEFL 15, Table 2.7). In both these cases, VOL is split into two groups, further complicating boundary placement. Optimal clustering (based on minimizing F_{SC}) when both temporal VOL samples are considered jointly included a boundary at the Flagler-Volusia County line in the case that northeastern Florida was not recognized as a discrete management unit (scenario NEFL 4), and inclusion of VOL and NSB as part of a recognized northeastern Florida management unit (scenario NEFL 13, Table 2.7).

Given the optimized boundaries for northeastern Florida considering separate treatment of the temporal VOL samples, a final round of AMOVA iterations was performed to test for optimal rookery clustering for the southeastern USA nesting aggregation. A total of five scenarios were considered given genetic evidence and inferences of rookery connectivity based on available demographic data and loggerhead turtle life history traits.

Scenario 1: Recognition of four management units: northern (Virginia through the Georgia-Florida border), peninsular Florida, Dry Tortugas, and northern Gulf (northwest Florida and westward). These are the currently recognized recovery units designated in the Recovery Plan (NMFS and USFWS 2008), and this scenario was considered a control.

Scenario 2: Recognition of six management units: northern, central eastern Florida, southern Florida (southeastern and southwestern), Dry Tortugas, central western Florida, and northwestern Florida

Scenario 3: Recognition of seven management units: northern, northeastern Florida, central eastern Florida, southern Florida (southeastern and southwestern), Dry Tortugas, central

western Florida, northwestern Florida

Scenario 4: Recognition of seven management units: northern, central eastern Florida, southeastern Florida, southwestern Florida, Dry Tortugas, central western Florida, and northwestern Florida

Scenario 5: Recognition of eight management units: northern, northeastern Florida, central eastern Florida, southeastern Florida, southwestern Florida, Dry Tortugas, central western Florida, and northwestern Florida

There was strong genetic structure among the discrete sample locations ($F_{ST} = 0.30325$, $p < 0.00001$) as well as among the management units tested in the five potential management scenarios (Table 2.8). AMOVA results indicated that a significant proportion (14.29%, $F_{SC} = 0.18863$, $p < 0.00001$; Table 2.8) of the overall genetic diversity of the southeastern USA nesting aggregation was partitioned among sampled rookeries within recovery units as they are currently recognized in the Recovery Plan. Although F_{SC} was reduced and F_{CT} was increased for all four remaining management schemes relative to the current Recovery Plan groupings, there was no clear best management scheme given the goal of maximizing F_{CT} and minimizing F_{SC}. Maximal F_{CT} was achieved with management scenario 2, recognition of central eastern, southern, and central western Florida management units from the current peninsular Florida recovery unit (Table 2.8). Minimal F_{SC} was achieved with management scenario 5, recognition of northeastern, central eastern, southeastern, southwestern, and central western Florida management units from the current peninsular Florida recovery unit (Table 2.8). A final round of pairwise F_{ST} comparisons and exact tests of population differentiation provided further support for recognition of the discrete management units outlined in scenario 2, with the only non significant comparison being that of central western and central eastern Florida across the axis of the Florida peninsula (Table 2.9). Southwestern Florida was not significantly different from the combined southeastern Florida rookeries in pairwise F_{ST} comparisons or exact tests of population differentiation (Tables 2.11 and 2.12).

Northeastern Florida was significantly different from proximal rookery clusters (Tables 2.10 and 2.12).

Discussion

Population Structure

The present study identified a pattern of haplotype frequency transitions that is generally consistent with earlier analyses that detected decreasing frequencies of haplotype CC-A1 and increasing frequencies of CC-A2 from north to south (Encalada et al. 1998; Bowen et al. 2005). However, the haplotype frequency patterns observed in the present study suggest an alternative interpretation to that of continuous, clinal variation in CC-A1 and CC-A2 along the Atlantic coast of Florida. Although there is an apparent cline across northeastern Florida rookeries, CAN and MEL2, separated by approximately 90 beach kilometers along the central coast of eastern Florida, had nearly identical and not significantly different haplotype frequencies. Similarly, the southeastern Florida sites, spanning roughly 125 kilometers (JUN through MID), had quite similar and not significantly different haplotype frequencies. Yet the frequencies of CC-A1 and CC-A2 are essentially inverted between MEL and JUN, which are separated by approximately 135 kilometers, a distance comparable to that spanning the southeastern Florida sites. The lack of a standard yardstick of geographic isolation that might predict genetic differentiation is echoed in the structure among loggerhead turtle rookeries in the Mediterranean basin. For instance, the sampled Greek rookeries of Zakynthos, Kyparissia, and Lakoninkos, each separated from the others by 100 km or more all shared nearly identical frequencies of haplotypes CC-A2 and CC-A6 (Encalada et al. 1998; Carreras et al. 2007). Yet the eastern Turkey and northern Cyprus rookeries, separated by approximately 100 km, had significantly different haplotype frequencies owing to the presence of CC-A3 at high frequency at the former and the absence of CC-A3 at the latter (Laurent et al. 1998; Carreras et al. 2007). Another similarity between southeastern USA and Mediterraean loggerhead

turtle nesting aggregations is the inference of a cline in the frequencies of CC-A2 and CC-A3 along the Turkish coast (Schroth et al. 1996; Carreras et al. 2007) that may mirror the observed cline in northeastern Florida. The broad nature of the apparent cline across northeastern Florida may have arisen out of the disparity in nesting densities between the northern management unit and the central eastern Florida rookeries (NMFS and USFWS 2008). Even a small proportion of females straying northward from central eastern Florida beaches would have a significant impact on haplotype frequencies given the nearly complete lack of CC-A2 individuals among northern rookeries.

The haplotype frequency transition patterns observed along the Atlantic coast of Florida suggests that rather than displaying broad clinal variation over the entire region, haplotype frequencies may be reasonably stable over 100 km. Such a pattern may result from female natal homing at sufficiently fine scales to maintain the frequency divergence between central and southern regions of Florida. The probability that a female strays to a non-natal site may not simply be a function of distance. Nesting females may be honing in on specific bathymetric (Mortimer 1982; Provancha and Ehrhart 1987), or other physical or chemical cues (Lohmann et al. 2008a) that could give rise to observed nesting density distribution patterns. Spatial analysis of 17 years of nesting density distribution data from the Florida Index Nesting Beach Survey program has revealed remarkable conservation of fine-scale nesting density patterns across nesting seasons (Witherington et al. 2009).

The strong divergence between central and southern Florida rookeries may reflect independent colonization of these areas. Encalada et al. (1998) hypothesized that an equatorial lineage (precursor to CC-A1, formerly haplotype A) may have colonized more northerly latitudes (into the Caribbean) prior to ultimately colonizing both the western and eastern coasts of Florida. Bowen et al. (1994) hypothesized that the CC-A2 lineage may have invaded the western Atlantic via southern Africa. Haplotype CC-A2 is the dominant haplotype in the Quintana Roo, Mexico loggerhead turtle rookery (55%, Encalada et al. 1998) as well as the most frequent haplotype among analyzed Cuban rookeries (Ruiz-Urquiola et al. 2010), so

colonization may have proceeded from either of these rookeries to southern Florida. One possible scenario is that the current nesting density peaks in Brevard (represented by MEL) and northern Palm Beach (represented by JUN) counties (NMFS and USFWS, 2008) represent sites that were initially colonized independently (perhaps originally by CC-A1 and CC-A2 lineages, respectively) and that the intervening beaches were colonized via diffusive natal dispersal from these core areas. Another possibility is that the region was initially colonized by the CC-A1 lineage and that the CC-A2 lineage represents a more recent colonization event. Given thermal constraints on incubation, the rookeries of northwestern and northeastern Florida and northward along the eastern coast of the USA most likely arose via recent colonization events since the Wisconsin glaciation (Encalada et al. 1998). Encalada et al. (1998) predicted that more recently colonized (more northerly) nesting areas would harbor decreasing haplotype diversity as haplotypes were sorted through a series of colonization bottlenecks. Whereas this is consistent with observations for the rookeries in northwestern Florida and northeastern Florida through North Carolina, the pattern did not hold for the southern sampled rookeries in the present study. The highest haplotype diversity was generally recorded at rookeries of intermediate latitude (CSK, JUN, and MEL) rather than those in southernmost Florida, suggesting the possibility that the more southern sites may have been colonized recently. Another possibility is that these high-density nesting beaches (relative to each respective region) have higher haplotype diversity by virtue of specific physical attributes which might attract nesting females carrying rare haplotypes that have strayed from other rookeries in the western Atlantic. While it is clear that at least two independent colonizations of the southeastern USA from external refugia occurred, it is uncertain whether the Gulf and Atlantic coasts of Florida were independently colonized from refugia or whether founders for novel rookeries on one coast may have originated from the other. Poor resolution of the mitochondrial marker does not permit unequivocal determination of the colonization pathways for the various rookeries comprising the nesting effort in the southeastern USA and requires more extensive screening of the mitochondrial genome for informative variation.

A striking feature of the haplotype frequency distribution is the slightly skewed mirror image pattern produced by comparable haplotype frequencies occurring at roughly similar latitudes across the Florida peninsula. One possible explanation for the overall pattern is error in natal homing that would compel females to nest on beaches with magnetic signatures similar to their natal beaches but on opposing coastlines across the Florida peninsula. Neonate marine turtles may imprint on the geomagnetic signature of their natal site and use this positional information to home to natal regions for nesting (Lohmann et al. 2008b). Marine turtles are sensitive to both magnetic inclination and intensity (Lohmann et al. 2007), however navigation utilizing a bicoordinate map may not be required to locate beaches along continental coastlines. The coastline itself may serve as a fixed coordinate; therefore turtles in search of natal regions would only need to follow the coastline to an appropriate inclination or intensity angle (Lohmann et al. 2008b). Tag returns demonstrate that some proportion of central eastern Florida nesting loggerhead turtles enter the Gulf of Mexico to forage (Meylan et al. 1983). Similarly, satellite telemetry indicated that six of twenty-eight females nesting in Sarasota County on the Gulf coast left the Gulf of Mexico following nesting to forage in the Bahamas (Girard et al. 2009). It is conceivable that a small proportion of females hatched on one coast of Florida but foraging off the other might inadvertently travel along the closest coastline and nest at a site with a similar one-dimensional magnetic signature as their natal area, but on the opposing coast across the axis of the Florida peninsula.

Nesting dispersal by individual females among rookeries as measured through flipper tagging studies may provide an alternative means of characterizing the magnitude and spatial scale of female gene flow among rookeries. For instance, extensive supplemental tagging of nesting Australian green turtles during the 1998-1999 nesting season revealed 8.3% interseasonal dispersal among southern Great Barrier Reef rookeries and 6% interseasonal dispersal among northern Great Barrier Reef rookeries, whereas no dispersal between southern and northern Great Barrier Reef rookeries was detected (Dethmers et al. 2006). The tagging observations were concordant with mtDNA analysis suggesting that rookeries within each

region were not genetically differentiated, but that the two regions represented distinct management units (Dethmers et al. 2006). Unfortunately, MEL currently hosts the only loggerhead turtle tagging project along the eastern coast of Florida, therefore contemporary data documenting west coast and east coast Florida nesting dispersal are scarce. Of thousands of loggerhead turtles tagged at CSK and at MEL since the mid 1980s, only seven have been recorded nesting at both of these sites (Mote Marine Laboratory and University of Central Florida Marine Turtle Research Group, unpubl. data). If effective, this level of migration is theoretically sufficient to prevent genetic differentiation of these rookeries (eg. Slatkin 1987).

A limitation of nesting beach flipper-tagging studies for rookery connectivity inference is that such studies measure nest site fidelity, the relative placement of nests by an individual female after she has been tagged while nesting, also known as site fixity or site tenacity (Carr and Carr 1972), rather than explicitly measuring natal philopatry (where the female nests relative to where she herself hatched). Nesting dispersal between distant rookeries represents natal dispersal by default, as it is illogical that a turtle could have hatched in two different regions. However, it is also conceivable that females exhibiting high site tenacity at a particular rookery could be nesting at a non-natal site (high nest site fidelity but low natal site fidelity). This type of natal dispersal would not be detectable with the tagging methodologies currently employed in the southeastern USA. Therefore, testing the hypothesis of inter-coastal natal dispersal within Florida in the absence of nesting dispersal will require a means of directly linking nesting females to their natal beaches.

Sample Sizes and Sampling Error

Small sample sizes and resulting sampling error likely contributed to underestimation, and in a few cases overestimation, of population differentiation. Despite complete sharing of haplotype CC-A1 between CAP ($n = 73$) and NC ($n = 43$), pairwise exact tests of population differentiation between these sites and all others yielded 19 and 17 significant comparisons, respectively. Sampling error by virtue of overestimation of the frequency of rare haplotypes

in a particular rookery based on a sample may also lead to differing conclusions regarding genetic divergence. For instance, the ten-day nest sample at STJ yielded individuals carrying three rare haplotypes absent among the much larger sample of nesting females from CSB (on the same peninsula, < 20 km away) obtained through saturation sampling over a period of four years. Larger sample sizes, particularly from sites with low nesting densities, will be required from many areas to make robust inferences regarding the possibility of additional management units within the southeastern United States nesting aggregation.

Temporal Variation in Haplotype Frequencies

It is unclear whether the apparent differentiation detected among year classes at CSB and MEL truly represents temporal variation or could have arisen through sampling error. The sample size of CSB1 was small (n = 7), and sampling methodology was unclear. Haplotype frequencies of CSB1 were significantly different from 2002 and 2004, whereas none of the remaining annual samples differed from one another, suggesting that CSB1 was an outlier that may have arisen through sampling error. MEL1 represents only a portion of turtles sampled (40 samples sequenced of 150 samples collected for a multiple paternity study, [Moore and Ball 2002]), so the difference between MEL1 and MEL2 may also be attributable to sampling error. Because of the high nesting densities at MEL (> 450 nests/km; NMFS and USFWS 2008), neither the 1996 nor the 2006 sample set represent strong sampling effort relative to nesting effort. The differentiation among annual VOL samples, however, does appear to truly reflect temporal variation given that sampling effort was high relative to nesting effort ($> 70\%$ of clutches laid during each sampling period) and sampling methodologies were consistent among years.

Lack of temporal variation at most sample sites is not surprising given the short duration between sampling periods and the estimated loggerhead turtle generation length of approximately 50 years (NMFS and USFWS 2008). Tag recoveries suggest that individual females are capable of nesting over a period of at least 25 years (NFMS and USFWS 2008).

Thus, any divergence in haplotype frequency via genetic drift would be expected to occur gradually as neophyte females are absorbed into the nesting population, slowly replacing senescent females. Bjorndal and Bolten (2008) argued that aggregates of females nesting at a rookery each year are probably well mixed due to individual females switching between remigration intervals of two, three, or more years (eg. Carr et al. 1978), likely maintaining genetic homogeneity among years.

If the apparent temporal variation observed at VOL is real, there are several alternative hypotheses worth considering. One possibility is that this variation is interannual and could be driven by differential aggregate mixing based on divergent foraging habitat use and differing mean remigration intervals for each foraging aggregation. Given the energetic costs of undertaking reproductive migrations and producing several clutches of eggs over the course of a nesting season, ecological conditions on the foraging grounds have been postulated to affect variability in remigration intervals (Carr and Carr 1970). Tröeng and Chaloupka (2007) hypothesized that the shorter observed population average remigration interval for Tortuguero green turtles relative to that of many other green turtle rookeries could be attributable, at least in part, to greater forage availability, better forage quality, and shorter distance between the nesting beach and the main foraging ground. Satellite telemetry and tag return data suggest that northern management unit loggerhead turtle females forage primarily along the continental shelf of the eastern United States, with a relatively small proportion of females moving south of the Cape Canaveral area to forage in the northern Caribbean or Gulf of Mexico (Bell and Richardson 1978; Plotkin and Spotila 2002; Williams and Frick 2008). Loggerhead turtles nesting in central eastern and western Florida typically forage in the Gulf of Mexico or in the northern Caribbean region (Meylan et al. 1983; Dodd and Byles 2003; Foley et al. 2008; Girard et al. 2009; Turtle Expert Working Group 2009), and only one satellite-tagged female has been recorded foraging north of the Cape Canaveral area (Dodd and Byles 2003).

Another possibility is that temporal variation exists within a nesting season. The initiation of nesting by central Florida and northern management unit females could be sufficiently staggered to produce cyclical changes in haplotype frequencies depending on the precise placement of the sampling window within the nesting season. Beyond different usage patterns of spatially discrete neritic habitats suggested by tag return and satellite telemetry data, analyses of stable isotopes and epibiota suggested that loggerhead turtles nesting along the eastern coast of Florida may be utilizing both oceanic and neritic foraging habitats (Reich et al. 2010). Although observed latitudinal trends in mtDNA haplotypes and stable isotope patterns were independent (Reich et al. 2010), the possibility remains that divergent foraging strategies or use of different foraging habitats could be driving sufficiently staggered nesting phenology for representatives of each group so as to cause temporal variation of haplotype frequencies on the nesting beach. Further research is warranted to determine whether haplotype frequency variations occur across individual nesting seasons at the northeastern Florida rookeries.

We concur with Bjorndal and Bolten (2008) that temporal variation should be considered in population structure analyses of rookeries as well as mixed stock analyses of foraging aggregations. Apparent temporal variation in the VOL rookery clearly had a significant impact on the interpretation of spatial genetic structure. The 1998 and 2007 samples would have lead to grouping of this rookery with those in central eastern Florida, whereas the 2006 and 2008 samples indicated a much closer affiliation with the northern management unit. Overall, these data suggest that a geographic transition zone occurs between the nesting populations in northeastern Florida and central eastern Florida. The apparent temporally transitional nature of haplotype frequencies at this rookery would have gone undetected without sampling over multiple years. Temporal variation of genetic diversity over short frames (eg. within a nesting season or less than a generation) may not occur as a rule at most rookeries, but should be considered particularly when rookeries may be suspected of being geographically transitional.

Defining Management Units

Defining management unit boundaries is inherently difficult when habitat is relatively homogenous and obvious barriers to movement are absent, such as the case of several hundred kilometers of essentially continuous coastline that provides suitable nesting habitat for loggerhead turtles. In cases where nesting habitats are discrete (e.g., Dry Tortugas) or are separated from other nesting areas by over 100 kilometers of unsuitable nesting habitat (e.g., northwest Florida beaches relative to central western Florida), management unit assessments may be straightforward if proximal rookeries have significantly different haplotype frequencies. However, boundaries along continuous nesting habitat must be artificially imposed in the sense that some proportion of females will distribute nesting effort on both sides of designated boundaries. Despite this complication, ignoring the genetic structure among peninsular Florida nesting areas could lead to inadequate protection of demographically distinct rookeries as well as misinterpretation of nesting trends at finer spatial scales.

Although genetic studies have provided a reasonable first approximation for management unit assignments (Bowen et al. 1993, Encalada et al. 1998, Pearce 2001, Bowen et al. 2005, present study), some inherent limitations of haplotype frequency data bear consideration. Provided sampling has been conducted in such a way as to maximize sample sizes and minimize sampling error, a significant difference in haplotype frequencies implies some level of demographic independence (Avise 1995). However, lack of significant genetic differences does not necessarily confer contemporary demographic connectivity (Taylor and Dizon 1996). Demographic partitioning despite non-significance of haplotype frequency comparisons is possible due to lack of resolution of the genetic markers, shared evolutionary history, and potentially insufficient time for genetic drift to occur. Comparative evidence suggests that marine turtle mitochondrial DNA evolves more slowly than that of most other vertebrates, possibly attributable to long generation time and low metabolic rate (Avise et al. 1992). Therefore nesting populations may be demographically isolated despite a lack of any detectable genetic differentiation. Distinguishing between recent shared evolutionary heritage

in the absence of genetic drift, low levels of contemporary genetic connectivity sufficient to prevent genetic divergence, and contemporary demographic connectivity among rookeries is critical for management on ecological time scales.

Ultimately, marine turtle rookeries flourish or perish based on recruitment of nesting females to a particular rookery (Bowen et al. 2005). Female nesting at non-natal sites is critical for colonization of novel nesting areas over evolutionary time scales, but natal dispersal of small numbers of females among distant established rookeries may be demographically irrelevant over ecological time scales. The level of exchange required to prevent genetic differentiation is many orders of magnitude lower than that required to sustain a population ecologically and demographically (Avise 1992). Whereas a few migrants per generation may be sufficient to maintain genetic homogeneity (Slatkin 1993), demographic independence of two populations may be maintained if less than 10% of individuals disperse between the populations (Hastings 1993). Thus management unit inferences should be drawn in the context of life history characteristics and available demographic data rather than relying strictly on the statistical significance of population differentiation tests.

Recovery Unit Recommendations

The present study upholds the distinctiveness of the four currently recognized recovery units: northern, peninsular Florida, Dry Tortugas, and northern Gulf of Mexico. We concur with the argument that the northern Gulf coast nesting population should be treated as a separate recovery unit on the basis of geographic isolation and apparent genetic distinction from the proximal Gulf coast rookeries in central western and southwestern Florida (Encalada et al. 1998).

Sampling effort has not been spatially or temporally adequate to fully resolve the number or boundaries of recovery units within the southeastern USA loggerhead turtle nesting aggregation. However, the present study does suggest more structure among peninsular Florida rookeries than is reflected in the current Recovery Plan designations. Although the lack of

data from the rookeries between MEL and JUN in the present analysis limits inferences about the nature of haplotype frequency transitions along the entire length of the Atlantic coast of Florida, similarity of haplotype frequencies within each sampled region and strong divergence of haplotype frequencies between them suggest some level of demographic partitioning. Brevard County in the central portion of the eastern coast of Florida and Palm Beach County in southeastern Florida host the two significant peaks in nesting density of the southeastern USA nesting aggregation with a relative trough of nesting densities between them (NMFS and USFWS 2008; Witherington et al., 2009). Given the nesting density distribution data and the significant genetic differentiation between the central and southern portions of the eastern coast, we recommend recognition of the central eastern Florida rookery as a distinct recovery unit. Similar genetic divergence occurs along the Gulf coast between KEY in southwestern Florida and CSK in central western Florida, and suggests that recognition of a separate central western Florida recovery unit is also warranted.

It is unclear whether the lack of genetic divergence between turtles nesting on the southernmost eastern and western coasts of the Florida peninsula reflects contemporary demographic connectivity, contemporary genetic connectivity, or may result from historical colonization signature. The discontinuity of suitable nesting habitat around the tip of the Florida peninsula (e.g. Davis and Whiting 1977), the scale of distinct management units inferred from the present study in other regions of Florida, and limited observed nesting dispersal between coasts suggest that each coast likely hosts demographically distinct rookeries. Though there is little genetic support for recognition of discrete southwestern and southeastern recovery units given the lack of significant differences of haplotype frequencies at KEY and the southeastern Florida rookeries, the conservative approach may be designation of finer scale recovery units unless or until evidence of sufficient effective movement between them is established. Further studies should address the demographic rookery connectivity between these regions.

The northeastern Florida rookeries present a challenge for recovery planning given the lack of a clear boundary between the northern and proposed central eastern Florida recovery

units because of intermediate haplotype frequencies. Pairwise F_{ST} comparisons, exact tests, and AMOVA results support the recognition of a discrete northeastern Florida recovery unit. However, the transitional nature of the haplotype frequencies of northeastern Florida rookeries both spatially and temporally in the case of VOL suggests that rather than representing a discrete nesting population, these rookeries represent a transition zone comprised of nesting females from both the northern and proposed central eastern Florida recovery units. Given the large disparity between nesting densities at Georgia rookeries and rookeries in central eastern Florida (10 20 nests/km versus 300 + nests/km, respectively; NMFS and USFWS 2008), even a small proportion of central eastern Florida straying northward into northeastern Florida would produce intermediate frequencies of CC-A2 relative to the rookeries to the north and south. Optimal boundaries for a discrete northeastern Florida recovery unit were between AML and SJC to the north and between VOL and NSB to the south when VOL temporal samples were treated discretely. Therefore, the Ponce Inlet may serve as an appropriate northern boundary for the central Florida recovery unit given either recognition of a northeastern Florida recovery unit or absorption of these northeastern Florida rookeries into the northern recovery unit. Under either scenario, AML should be treated as part of the northern recovery unit based on AMOVA results. Further research should focus on the demographic independence of the northeastern Florida rookeries relative to those in Georgia and central eastern Florida.

The genetic data support recognition of a minimum of six distinct recovery units: northern, central eastern Florida, southern Florida (southeastern and southwestern), Dry Tortugas, central western Florida, and northern Gulf of Mexico. The demographic discreteness of northeastern and southwestern Florida rookeries is unclear and warrants further research. More extensive genetic sampling is required to fill geographic gaps in the present study and to better describe the nature of haplotype frequency transitions along continuous coastlines. Further demographic partitioning likely occurs, and additional tools, such as satellite or GPS telemetry and mark-recapture, are required to overcome the limitations of

the mitochondrial sequence data used in the present study in generating more robust data on the scale of female natal philopatry, female nest site fidelity, and connectivity among rookeries.

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Table 2.1: Sample site and collection data for samples collected as part of the present study.

Site Code	Sample Site	Sample Size	Year	Sample Type
BHI	Bald Head Island, Brunswick County, North Carolina	15	2006	female[a]
CAP	Cape Island, Charleston County, South Carolina	53	2006	female
WAS	Wassaw Island, Chatham County, Georgia	42	2005, 2006	female
AML	Amelia Island, Nassau County, Florida	20	2006, 2008	nest[b]
SJC	St. Johns County, Florida	37	2007, 2008	nest
FLG	Flagler County, Florida	55	2007, 2008	nest
VOL	northern Volusia County, Florida	90	2006-2008	nest
NSB	New Smyrna Beach, Volusia County, Florida	46	2006, 2008	nest
CAN	Canaveral National Seashore, Volusia County, Florida	58	2006	female
MEL	Melbourne Beach, Brevard County, Florida	106	2006	female
JUN	Juno Beach, Palm Beach County, Florida	49	2006	female
FTL	Ft. Lauderdale, Broward County, Florida	48	2006	nest
SBR	Hollywood and John U. Lloyd State Park, Broward County, Florida	21	2006	nest
MID	Virginia Key and Cape Florida State Park, Miami-Dade County, Florida	22	2006	nest
KEY	Keewaydin Island, Collier County, Florida	40	2006	female
CSK	Casey Key, Sarasota County, Florida	57	2006	female
SGI	St. George Island, Franklin County, Florida	13	2006	nest
CSB	Cape San Blas, Gulf County, Florida	47	2002-2005	female
STJ	St. Joseph Peninsula State Park, Gulf County, Florida	15	2006	nest

[a]Female samples were collected as biopsy punches

[b]Nest samples were dead hatchling tissue or hatched egg shells

Table 2.2: Variable positions of a 390 base pair fragment of the mitochondrial control region observed in loggerhead turtles nesting in the southeastern USA. Haplotype designations are based on Archie Carr Center for Sea Turtle Research nomenclature. '-' indicates a deletion.

	Variable Positions																														
	3	3	3	5	5	6	7	9	1 0	1 1	1 6	1 6	1 8	2 1	2 3	2 4	2 4	2 5	2 9	3 1	3 1	3 1	3 1	3 2	3 5	3 5	3 5	3 5	3 5	3 6	3 6
Haplotype	2	5	7	1	3	3	8	6	4	9	1	2	8	0	0	4	6	9	4	2	4	5	7	7	5	6	7	8	9	0	3
CC-A2	C	A	C	C	-	G	G	A	G	A	T	A	G	T	T	A	T	G	G	A	G	C	G	T	G	C	A	A	G	T	A
CC-A3	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A5[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
CC-A7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
CC-A9	.	.	.	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
CC-A13[a]	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A20	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A43	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A1	T	G	T	T	T	A	.	.	A	.	C	G	.	C	C	G	C	A	-	.	A	T	A	C	-	-	-	-	-	-	.
CC-A11[a]	T	G	T	T	T	A	.	.	A	.	C	.	.	C	C	G	C	A	-	.	A	T	A	C	-	-	-	-	-	-	.
CC-A14	T	G	T	T	T	A	A	.	A	.	C	.	.	C	C		C	A	-	.	A	T	A	C	-	-	-	-	-	-	.

[a]Previously described haplotypes from Florida rookeries not detected in the present study

Table 2.3: Haplotype composition of loggerhead turtle rookeries from North Carolina to northeastern Florida and within sample haplotype diversity (h) and nucleotide diversity (π). Sample site abbreviations are included in Table 2.1 for samples collected as part of the present study and in the footnotes for previously collected samples representing beaches not defined in Table2.1. "CC-A" haplotype prefixes are excluded for legibility.

		Haplotype														
Site	Year[a]	A1	A2	A3	A5	A7	A9	A10	A11	A13	A14	A20	A43	N	h	π
NC[b, e]	1987-1993	28												25	0.000 ± 0.000	0.0000 ± 0.0000
BHI	2006	15												15	0.000 ± 0.000	0.0000 ± 0.0000
CAP[b]	1987-1993	20												20	0.000 ± 0.000	0.0000 ± 0.0000
CAP	2006	53												53	0.000 ± 0.000	0.0000 ± 0.0000
WAS	2005	8												8	0.000 ± 0.000	0.0000 ± 0.0000
WAS	2006	34												34	0.000 ± 0.000	0.0000 ± 0.0000
SGA[b,f]	1987-1991	42	1											43	0.047 ± 0.044	0.0022 ± 0.0018
AML[b]	1987-1993	12												12	0.000 ± 0.000	0.0000 ± 0.0000
AML	2006	8	1											9	0.222 ± 0.166	0.0119 ± 0.0073
AML	2008	11												11	0.000 ± 0.000	0.0000 ± 0.0000
SJC	2007	22	3	1							1			27	0.333 ± 0.111	0.0134 ± 0.0075
SJC	2008	10												10	0.000 ± 0.000	0.0000 ± 0.0000
FLG	2007	27	5											32	0.272 ± 0.089	0.0132 ± 0.0073
FLG	2008	20	2	1										23	0.245 ± 0.113	0.0117 ± 0.0067
VOL[c]	1998	23	9	1				1		1				35	0.514 ± 0.078	0.0228 ± 0.0120
VOL	2006	20		1		1								22	0.178 ± 0.106	0.0089 ± 0.0053
VOL	2007	23	7	1										31	0.421 ± 0.087	0.0197 ± 0.0106
VOL	2008	32	2	1							2			37	0.252 ± 0.092	0.0083 ± 0.0049
NSB[c]	1998	9	4					1						14	0.539 ± 0.115	0.0240 ± 0.0132
NSB	2006	19	2											21	0.181 ± 0.104	0.0087 ± 0.0052
NSB	2008	19	4	1		1								25	0.410 ± 0.111	0.0188 ± 0.0102
CAN	2006	38	17	1		1					1			58	0.492 ± 0.054	0.0220 ± 0.0115
MEL[b]	1987-1993	5	1											6	0.333 ± 0.215	0.0161 ± 0.0103
MEL[d]	1996	20	16			1			1		1	1		40	0.603 ± 0.046	0.0248 ± 0.0129
MEL	2006	74	27	3							2			106	0.451 ± 0.044	0.0201 ± 0.0105

[a]Collection year ranges are presented for each data set from the literature where precise years are unknown

[b]Haplotype frequency data from Encalada et al. 1998

[c]Haplotype frequency data from Francisco et al. 1999 and Bowen et al. 2005

[d]Haplotype frequency data from Pearce 2001 and Bowen et al. 2005

Table 2.4: Haplotype composition of southeastern Florida and Gulf of Mexico and within sample haplotype diversity (h) and nucleotide diversity (π). Sample site abbreviations are included in Table 2.1 for samples collected as part of the present study and in the footnotes for previously collected samples representing beaches not defined in Table 2.1. "CC-A" haplotype prefixes are excluded for legibility.

		Haplotype														
Site	Year[a]	A1	A2	A3	A5	A7	A9	A10	A11	A13	A14	A20	A43	N	h	π
JUN	2006	9	29	5		1		1			1	2	1	49	0.615 ± 0.069	0.0169 ± 0.0090
FTL	2006	14	32	2										48	0.479 ± 0.058	0.0206 ± 0.0108
PEV[b,d]	1987-1993	3	6		1									10	0.600 ± 0.131	0.0226 ± 0.0129
SBR	2006	5	16											21	0.381 ± 0.101	0.0184 ± 0.0101
MID	2006	6	16											22	0.416 ± 0.090	0.0200 ± 0.0109
DRT[c,e]	1995-1999	4	50				2	2						58	0.254 ± 0.074	0.0068 ± 0.0041
KEY[b]	1987-1993	5	9	1										15	0.562 ± 0.095	0.0234 ± 0.0128
KEY	2006	17	22									1		40	0.530 ± 0.035	0.0242 ± 0.0126
SAR[b,f]	1987-1993	5	3	1		1								10	0.711 ± 0.118	0.0280 ± 0.0158
SAR[c,f]	1995-1999	10	5	2		1					1			19	0.673 ± 0.090	0.0261 ± 0.0140
CSK	2006	35	13	5			1	1			2			57	0.571 ± 0.061	0.0232 ± 0.0121
SGI	2006	11	1	1										13	0.295 ± 0.156	0.0141 ± 0.0082
CSB[c]	1995-1999	4	3											7	0.571 ± 0.120	0.0276 ± 0.0164
CSB	2002	14					1							15	0.133 ± 0.112	0.0064 ± 0.0041
CSB	2003	10	1	1										12	0.318 ± 0.164	0.0151 ± 0.0088
CSB	2004	10												10	0.000 ± 0.000	0.0000 ± 0.0000
CSB	2005	9	1											10	0.200 ± 0.154	0.0097 ± 0.0060
STJ[c]	1987-1993	9	2											11	0.327 ± 0.153	0.0158 ± 0.0092
STJ	2006	12				1		1			1			15	0.371 ± 0.153	0.0132 ± 0.0077

[a]Collection year ranges are presented for each data set from the literature where precise years are unknown.

[b]Haplotype frequency data from Encalada et al. 1998

[c]Haplotype frequency data from Pearce 2001 and Bowen et al. 2005

[d]Port Everglades, Broward County, Florida

[e]Dry Tortugas, Monroe County, Florida

Table 2.5: AMOVA results for within-rookery tests of interannual haplotype frequency variation. Proportion TV is the percentage of total genetic variation within each pooled rookery sample set explained by interannual variation in haplotype frequencies.

Rookery	F_{ST}	P value	Proportion TV
GA	-0.02163	0.49901	-2.16
AML	0.02778	0.28257	2.78
SJC	0.03025	0.29366	3.03
FLG	-0.02382	0.68050	-2.38
VOL	0.05002	0.02257	5.00
NSB	0.02030	0.24000	2.03
MEL	0.03680	0.07782	3.68
SBR	-0.03009	0.46901	-3.01
KEY	-0.03198	0.66327	-3.20
CSK	-0.03322	0.94059	-3.32
CSB	0.10958	0.02950	10.96
STJ	-0.01278	0.40267	-1.28

Table 2.6: Pairwise F_{ST} values for discrete rookery sample comparisons (above the diagonal) and p values of exact tests of population differentiation (below the diagonal). Significant pairwise F_{ST} comparisons ($\alpha = 0.05$, no correction for multiple tests) are indicated in bold.

	NC	CAP	GA	AML	SJC	FLG	VOL1	VOL2	NSB	CAN	MEL1	MEL2	JUN	FTL	SBR	MID	DRT	KEY	CSK	SGI	CSB1	CSB2	STJ
NC		0.000	-0.009	0.009	**0.081**	**0.098**	**0.046**	**0.201**	**0.136**	**0.255**	**0.407**	**0.185**	**0.608**	**0.649**	**0.739**	**0.791**	**0.846**	**0.527**	**0.225**	0.181	**0.713**	0.038	**0.130**
CAP	0.9990		-0.002	0.029	**0.120**	**0.134**	**0.069**	**0.250**	**0.177**	**0.314**	**0.488**	**0.220**	**0.675**	**0.713**	**0.801**	**0.849**	**0.878**	**0.596**	**0.269**	0.273	**0.804**	**0.062**	**0.191**
GA	0.9990	1.0000		-0.010	**0.089**	**0.109**	**0.052**	**0.238**	**0.159**	**0.302**	**0.481**	**0.210**	**0.675**	**0.709**	**0.791**	**0.833**	**0.870**	**0.592**	**0.263**	0.180	**0.738**	0.036	**0.156**
AML	0.4473	0.3048	0.4745		0.016	0.035	0.006	**0.136**	**0.073**	**0.182**	**0.317**	**0.131**	**0.537**	**0.575**	**0.656**	**0.699**	**0.800**	**0.450**	**0.170**	0.035	**0.500**	-0.009	0.048
SJC	0.0147	0.0031	0.0124	0.6174		-0.017	-0.016	0.048	0.002	**0.083**	**0.199**	**0.052**	**0.437**	**0.463**	**0.524**	**0.545**	**0.710**	**0.340**	**0.085**	-0.047	0.210	-0.013	-0.021
FLG	0.0117	0.0012	0.0022	0.2533	0.6427		0.006	0.035	-0.006	**0.067**	**0.187**	**0.038**	**0.437**	**0.452**	**0.512**	**0.529**	**0.692**	**0.327**	**0.079**	-0.037	0.177	0.004	-0.012
VOL1	0.0420	0.0022	0.0167	0.8286	0.9261	0.1386		**0.089**	**0.031**	**0.130**	**0.262**	**0.090**	**0.497**	**0.522**	**0.583**	**0.604**	**0.738**	**0.403**	**0.123**	-0.035	0.294	-0.012	-0.008
VOL2	<0.0001	<0.0001	<0.0001	0.0162	0.1203	0.2470	0.0008		0.001	-0.010	0.044	-0.011	**0.263**	**0.259**	**0.301**	**0.302**	**0.514**	**0.151**	0.003	0.014	-0.025	**0.096**	0.024
NSB	0.0010	<0.0001	0.0002	0.1512	0.5205	0.8228	0.0332	0.7695		0.021	**0.114**	0.006	**0.359**	**0.365**	**0.415**	**0.423**	**0.614**	**0.246**	**0.037**	-0.024	0.065	0.037	-0.012
CAN	<0.0001	<0.0001	<0.0001	0.0031	0.0357	0.0403	0.0006	0.7812	0.3852		0.013	-0.009	**0.215**	**0.207**	**0.246**	**0.245**	**0.468**	**0.106**	-0.002	0.046	-0.057	**0.140**	0.050
MEL1	<0.0001	<0.0001	<0.0001	0.0004	0.0007	0.0006	<0.0001	0.0484	0.0035	0.4102		**0.045**	**0.091**	**0.081**	**0.107**	**0.103**	**0.329**	0.013	0.017	0.144	-0.083	**0.273**	**0.144**
MEL2	<0.0001	<0.0001	<0.0001	0.0089	0.0893	0.1446	0.0001	0.5723	0.2587	0.7459	0.0257		**0.270**	**0.259**	**0.300**	**0.300**	**0.498**	**0.152**	0.006	0.021	-0.025	**0.097**	0.030
JUN	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0065	<0.0001		0.002	0.005	0.003	**0.092**	0.030	**0.182**	**0.373**	0.070	**0.505**	**0.378**
FTL	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	0.0160	0.0006	0.2410		-0.021	-0.028	**0.099**	0.003	**0.186**	**0.410**	0.055	**0.535**	**0.406**
SBR	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0000	0.0276	<0.0001	0.3013	0.5061		-0.038	**0.068**	0.018	**0.220**	**0.470**	**0.095**	**0.604**	**0.459**
MID	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.0033	0.1536	0.0005	0.6941	1.0000	1.0000		**0.068**	0.012	**0.218**	**0.489**	0.094	**0.632**	**0.473**
DRT	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0012	0.0015	0.0218	0.1045		**0.195**	**0.416**	**0.697**	**0.411**	**0.759**	**0.670**
KEY	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	0.0008	<0.0001	0.0058	0.2299	0.0005	0.0303	0.4796	0.2452	0.6398	<0.0001		**0.101**	**0.287**	-0.037	**0.411**	**0.287**
CSK	<0.0001	<0.0001	<0.0001	0.0015	0.0615	0.0152	0.0004	0.1951	0.0432	0.2506	0.0423	0.0971	<0.0001	0.0000	0.0000	0.0028	<0.0001	0.0012		**0.042**	-0.042	**0.133**	**0.055**
SGI	0.0557	0.0213	0.0486	0.1979	0.8246	0.5718	0.6252	0.4508	0.5709	0.2486	0.0762	0.3370	0.0003	0.0001	0.0004	0.0006	<0.0001	0.0028	0.6460		0.134	-0.028	-0.042
CSB1	0.0020	0.0004	0.0008	0.0148	0.1098	0.1850	0.0348	0.5760	0.3819	0.7650	1.0000	0.5584	0.5431	0.3962	0.3234	0.1926	0.0089	0.5655	0.8236	0.1651		**0.342**	0.125
CSB2	0.1289	0.0193	0.0622	1.0000	0.8193	0.3461	0.7359	0.0098	0.1104	0.0014	<0.0001	0.0038	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0019	0.4767	0.0526		0.006
STJ	0.0049	0.0012	0.0020	0.2673	0.8146	0.1339	0.4607	0.0857	0.3673	0.0747	0.0074	0.0253	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0544	0.9155	0.2483	0.2773	

Table 2.7: Rookery-clustering scenario for northeastern Florida AMOVA tests. MU is management unit. Only northeastern Florida rookeries are shown in the table, but the northern MU also includes NC, CAP, and GA. The central eastern Florida MU also includes CAN, MEL1, MEL2.

	northern MU	northeastern Florida MU	central eastern FL MU
NEFL1			AML, SJC, FLG, VOL1, VOL2, NSB
NEFL2	AML		SJC, FLG, VOL1, VOL2, NSB
NEFL3	AML, SJC		FLG, VOL1, VOL2, NSB
NEFL4	AML, SJC, FLG		VOL1, VOL2, NSB
NEFL5	AML, SJC, FLG, VOL1		VOL2, NSB
NEFL6	AML, SJC, FLG, VOL2		VOL1, NSB
NEFL7	AML, SJC, FLG, VOL1, VOL2		NSB
NEFL8	AML, SJC, FLG, VOL1, VOL2, NSB		
NEFL9		AML, SJC, FLG, VOL1, VOL2, NSB	
NEFL10		AML, SJC, FLG, VOL1, VOL2	NSB
NEFL11		AML, SJC, FLG, VOL1	VOL2, NSB
NEFL12		AML, SJC, FLG	VOL1, VOL2, NSB
NEFL13	AML	SJC, FLG, VOL1, VOL2, NSB	
NEFL14	AML	SJC, FLG, VOL1, VOL2	NSB
NEFL15	AML	SJC, FLG, VOL1	VOL2, NSB
NEFL16	AML	SJC, FLG	VOL1, VOL2, NSB

Table 2.8: AMOVA results for rookery-clustering scenarios to resolve treatment of north-eastern Florida rookeries. P values for all F_{CT} indices were < 0.00001. AMU is the porportion of genetic variation partitioned among management units. AR/MU is the total proportion of genetic variance among sampled rookeries within management units.

	F_{CT}	AMU	F_{SC}	F_{SC} p	AR/WMU
NEFL 1	0.32519	32.52%	0.04274	<0.00001	2.88%
NEFL 2	0.32833	32.83%	0.03416	0.00010	2.29%
NEFL 3	0.32637	32.64%	0.03232	0.00020	2.18%
NEFL 4	0.32424	32.42%	0.03143	<0.00001	2.12%
NEFL 5	0.33606	33.61%	0.01647	0.00901	1.09%
NEFL 6	0.30743	30.74%	0.05166	<0.00001	3.58%
NEFL 7	0.32312	32.31%	0.03685	<0.00001	2.49%
NEFL 8	0.33042	33.04%	0.03603	0.00010	2.41%
NEFL 9	0.33061	33.06%	0.01735	0.02020	1.16%
NEFL 10	0.32502	32.50%	0.02292	0.00495	1.55%
NEFL 11	0.33761	33.76%	0.00986	0.09950	0.65%
NEFL 12	0.32984	32.98%	0.02788	0.00267	1.87%
NEFL 13	0.33281	33.28%	0.01287	0.04554	0.86%
NEFL 14	0.32772	32.77%	0.01907	0.01446	1.28%
NEFL 15	0.33946	33.95%	0.00847	0.11495	0.56%
NEFL 16	0.33296	33.30%	0.02620	0.00198	1.75%

Table 2.9: Pairwise F_{ST} values (above the diagonal) and exact test of population differentiation p values (below the diagonal) for final management unit groupings outlined in scenario 2. CE FL is central eastern Florida. S FL is southeastern and southwestern Florida. NW FL is northwestern Florida. Significant F_{ST} values and exact test p values following sequential Bonferroni correction with a table wide alpha of 0.05 are indicated in bold.

	northern	CE FL	S FL	DRT	CSK	NW FL
northern		**0.175**	**0.610**	**0.849**	**0.309**	**0.036**
CE FL	**<0.00001**		**0.231**	**0.456**	0.008	**0.056**
S FL	**<0.00001**	**<0.00001**		**0.093**	**0.180**	**0.405**
DRT	**<0.00001**	**<0.00001**	**0.00004**		**0.416**	**0.690**
CSK	**<0.00001**	**0.01209**	**<0.00001**	**<0.00001**		**0.093**
NW FL	**0.01056**	**0.00876**	**<0.00001**	**<0.00001**	**0.00158**	

Table 2.10: Pairwise F_{ST} values (above the diagonal) and exact test of population differentiation p values (below the diagonal) for final management unit groupings outlined in scenario 3. NE FL is northeastern Florida (SJC, FLG, and VOL1). CE FL is central eastern Florida. S FL is southeastern and southwestern Florida. NW FL is northwestern Florida. Significant F_{ST} values and exact test p values following sequential Bonferroni correction with a table wide alpha of 0.05 are indicated in bold.

	northern	NE FL	CE FL	S FL	DRT	CSK	NW FL
northern		**0.088**	**0.219**	**0.639**	**0.925**	**0.402**	**0.13**
NE FL	**<0.00001**		**0.074**	**0.445**	**0.718**	**0.125**	-0.007
CE FL	**<0.00001**	**0.00009**		**0.231**	**0.456**	0.008	**0.056**
S FL	**<0.00001**	**<0.00001**	**<0.00001**		**0.093**	**0.18**	**0.405**
DRT	**<0.00001**	**<0.00001**	**<0.00001**	**0.00003**		**0.416**	**0.690**
CSK	**<0.00001**	**<0.00001**	**0.00798**	**<0.00001**	**<0.00001**		**0.093**
NW FL	**<0.00001**	0.74327	**0.00674**	**<0.00001**	**<0.00001**	**0.00132**	

Table 2.11: Pairwise F_{ST} values (above the diagonal) and exact test of population differentiation p values (below the diagonal) for final management unit groupings outlined in scenario 4. CE FL is central eastern Florida. SE FL is southeastern Florida. NW FL is northwestern Florida. Significant F_{ST} values and exact test p values following sequential Bonferroni correction with a table wide alpha of 0.05 are indicated in bold.

	northern	CE FL	SE FL	DRT	KEY	CSK	NW FL
northern		**0.159**	**0.631**	**0.840**	**0.607**	**0.276**	**0.032**
CE FL	**<0.00001**		**0.265**	**0.456**	**0.145**	0.008	**0.056**
SE FL	**<0.00001**	**<0.00001**		**0.071**	0.020	**0.208**	**0.448**
DRT	**<0.00001**	**<0.00001**	**0.00134**		**0.195**	**0.416**	**0.690**
KEY	**<0.00001**	**0.00190**	0.59579	**<0.00001**		**0.100**	**0.357**
CSK	**<0.00001**	**0.00924**	**<0.00001**	**<0.00001**	**0.00013**		**0.093**
NW FL	0.02786	**0.00653**	**<0.00001**	**<0.00001**	**<0.00001**	**0.00155**	

Table 2.12: Pairwise F_{ST} values (above the diagonal) and exact test of population differentiation p values (below the diagonal) for final management unit groupings outlined in scenario 5. NE FL is northeastern Florida (SJC, FLG, and VOL1). CE FL is central eastern Florida. SE FL is southeastern Florida. NW FL is northwestern Florida. Significant F_{ST} values and exact test p values following sequential Bonferroni correction with a table wide alpha of 0.05 are indicated in bold.

	northern	NE FL	CE FL	SE FL	DRT	KEY	CSK	NW FL
northern		**0.088**	**0.219**	**0.702**	**0.925**	**0.742**	**0.402**	**0.13**
NE FL	**<0.00001**		**0.074**	**0.491**	**0.718**	**0.412**	**0.125**	-0.007
CE FL	**<0.00001**	**<0.00001**		**0.265**	**0.456**	**0.145**	0.008	**0.056**
SE FL	**<0.00001**	**<0.00001**	**<0.00001**		**0.071**	0.020	**0.208**	**0.448**
DRT	**<0.00001**	**<0.00001**	**<0.00001**	**0.00094**		**0.195**	**0.416**	**0.690**
KEY	**<0.00001**	**<0.00001**	**0.00143**	0.59846	**<0.00001**		**0.101**	**0.357**
CSK	**<0.00001**	**<0.00001**	**0.00727**	**<0.00001**	**<0.00001**	**0.00058**		**0.093**
NW FL	**<0.00001**	0.73712	**0.00779**	**<0.00001**	**<0.00001**	**<0.00001**	**0.00145**	

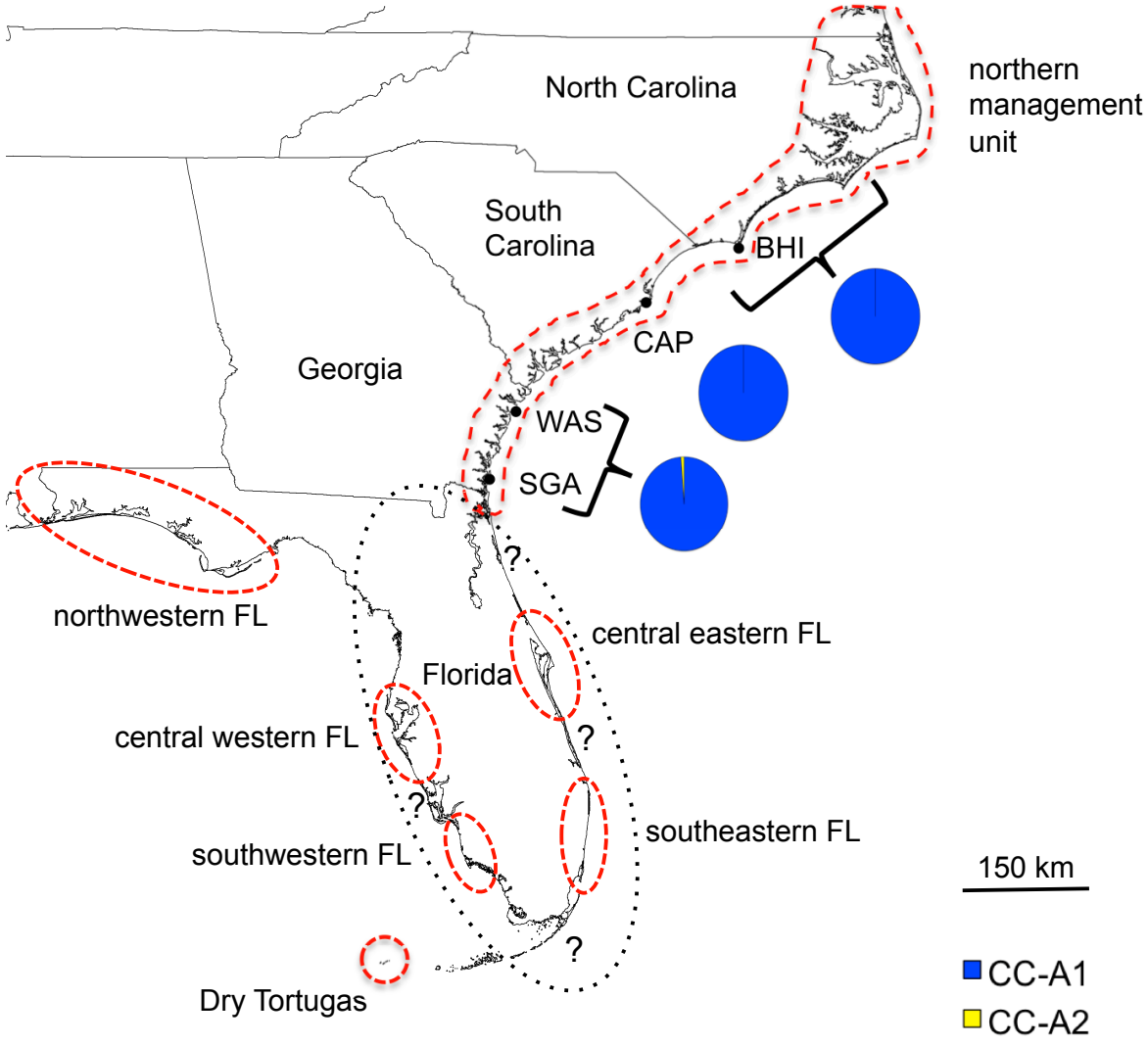

Figure 2.1: Sample locations and haplotype distributions for northern sampled rookeries of southeastern USA loggerhead turtles. Regional rookery groupings discussed in the text are outlined in dashed lines. The currently recognized peninsular Florida recovery unit is outlined by dotted line. See Fig 5.2 for Florida sample sites and haplotype frequency pie charts.

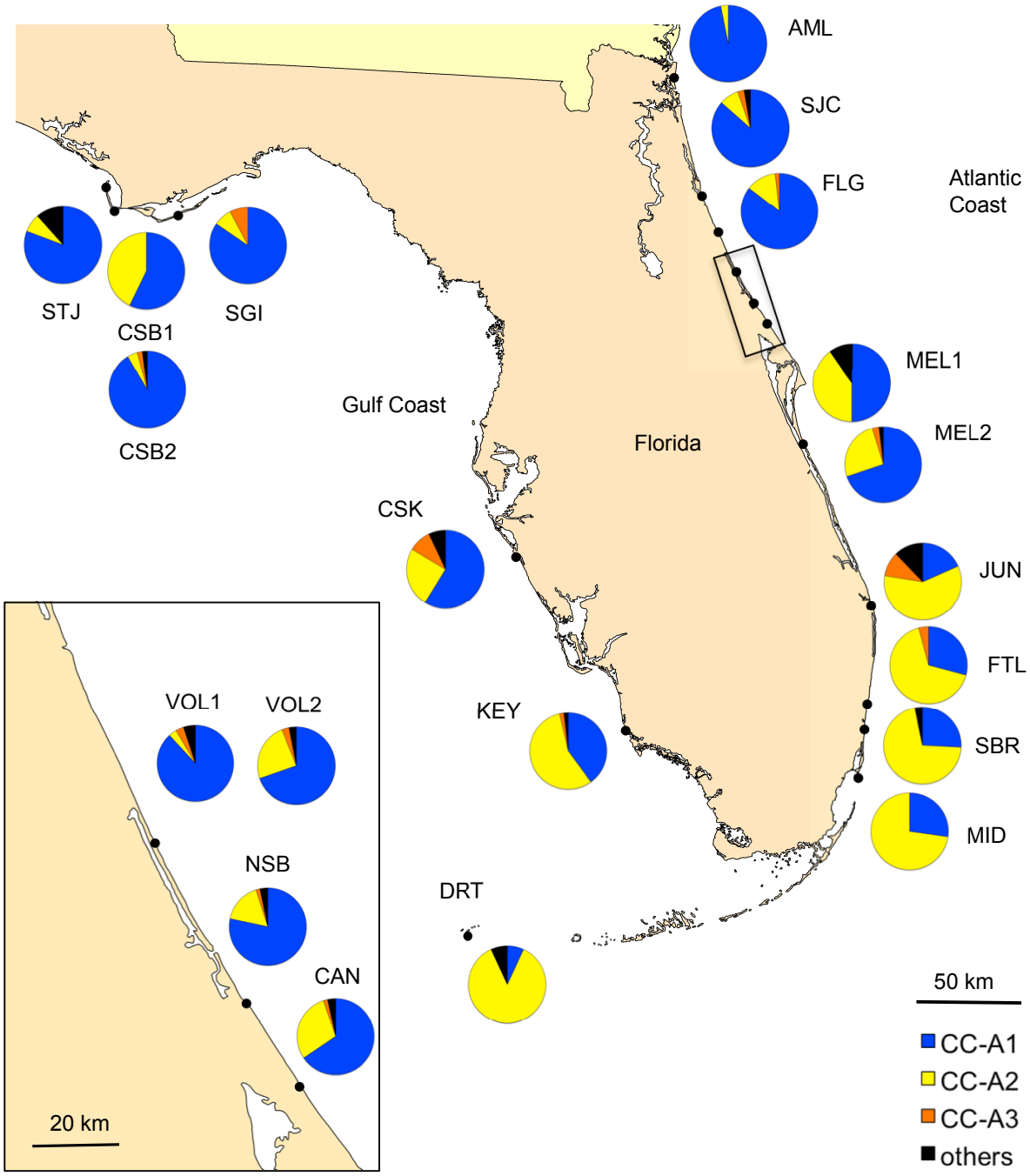

Figure 2.2: Sample locations and partial mitochondrial control region haplotype frequencies for Florida loggerhead turtle rookeries based on combined haplotype frequency data from the present study and previous studies. Site abbreviations are explained in Table 2.1. Northeastern Florida data are highlighted in the inset map.

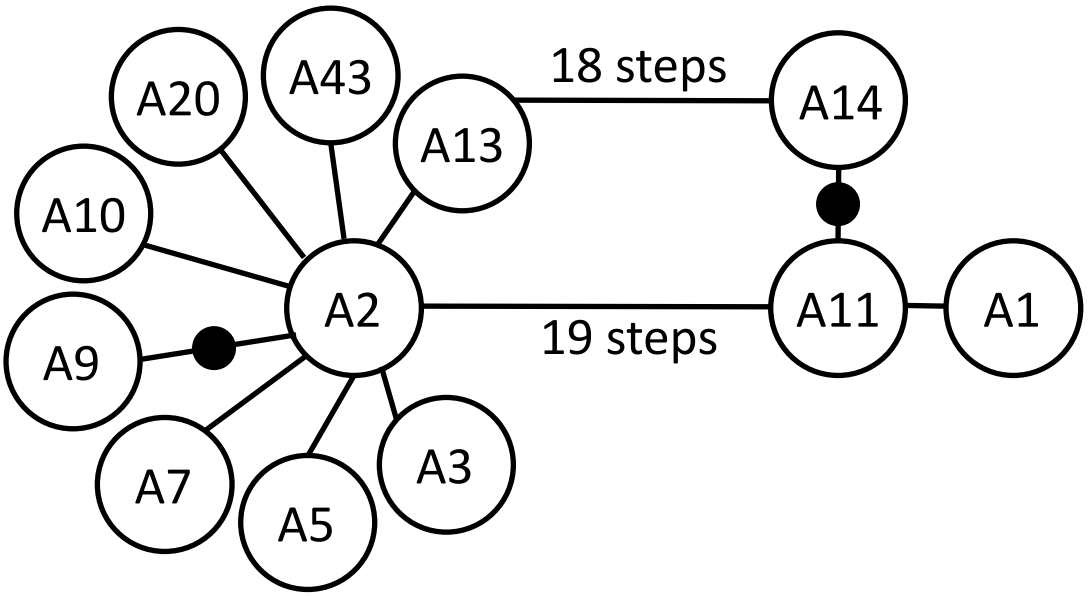

Figure 2.3: Haplotype network depicting the two phylogenetically distinct haplogroups previously described by Encalada et al. (1998). Filled circles indicate hypothetical haplotypes.

Chapter 3

Population structure of southeastern United States loggerhead turtle rookeries revisited with expanded mitochondrial control region sequences[1]

[1]Shamblin BM, Bjorndal KA, Bolten AB, Reich KJ, Dutton PH, LaCasella EL, Tucker AD, Bagley DA, Ehrhart LM, Witherington BE, Addison DL, Mota MJ, Carthy RR, Lamont MM, Dodd MG, Nairn CJ. To be submitted to Conservation Genetics.

Abstract

The southeastern United States of America (USA) hosts the largest concentration of loggerhead turtle (*Caretta caretta*) nesting in the Atlantic and one of two globally significant nesting aggregations. Previous studies based on a 390 base pair fragment of the mitochondrial control region supported recognition of six demographically independent nesting populations (management units) in the region. Recent analysis of expanded mitochondrial control region sequences indicated increased genetic diversity and stronger population structure between western and eastern Atlantic loggerhead turtle rookeries. In order to generate baseline data for mixed stock analyses and reassess population structure among southeastern USA rookeries, we sequenced an 817 base pair mitochondrial fragment in 2260 samples from twelve southeastern USA nesting beaches and Cay Sal Bank, Bahamas. Pairwise F_{ST} comparisons, pairwise exact tests of population differentiation, and analysis of molecular variance support previously proposed management unit designations and additionally indicate that southeastern and southwestern Florida rookeries should be recognized as distinct management units. Therefore the southeastern USA nesting aggregation can be subdivided into seven management units, corresponding to the beaches from: 1) Virginia through northeastern Florida, 2) central eastern Florida, 3) southeastern Florida, 4) Dry Tortugas (and Cay Sal, Bahamas), 5) southwestern Florida, 6) central western Florida, and 7) northwestern Florida.

Introduction

Defining population structure of vagile marine species is often complicated given the lack of apparent barriers to movement across vast spatial scales. For marine turtles, natal philopatry to specific reproductive sites dictates population boundaries (reviewed in Bowen and Karl 2007). Lohmann et al. (2008) proposed that both breeding salmon and marine turtles locate natal regions through a biphasic navigation process initially utilizing magnetic cues to direct long distance ocean migration to the general vicinity of the natal area. Salmon then use

local olfactory cues to direct navigation to specific spawning rivers (Wisby and Hasler 1954); however, the local cues driving fine scale nesting beach selection by marine turtles are less well understood (Lohmann et al. 2008). Therefore the precise scale of natal homing remains unclear. The presence of several hundred km of essentially continuous nesting habitat along continental coastlines further complicates assessments of population structure for marine turtles. Nonetheless, given that migratory reproductive behavior contributes significantly to patterns of population structure for these species, properly the defining the scale of natal homing behavior is critical to ensuring that demographically discrete populations receive adequate recognition and protection.

Loggerhead sea turtles are distributed in warm temperate waters circumglobally (Bolten 2003). The two largest nesting aggregations known occur in the southeastern United States of America (USA) and Masirah, Oman (Dodd 1988; Baldwin et al. 2003). In the southeastern USA, loggerhead turtles nest regularly where suitable sandy beaches occur from Virginia to Alabama. Nesting densities vary considerably along this coastline with just six counties in eastern Florida accounting for approximately 80% of nesting effort for the species nationally (NMFS and USFWS 2008). Annual mean nest numbers on Floridas index nesting beaches declined by approximately 44% from 1998 through 2006 (Witherington et al. 2009), prompting concern that the largest nesting population in the Atlantic may be in decline.

Haplotype frequency analyses of a 390 bp portion of the mitochondrial control region have suggested the presence of several management units for loggerhead turtles in the Atlantic and Mediterranean basins (Encalada et al. 1998; Bowen et al. 2004; Carreras et al. 2007; Ruiz U. et al. 2008; Garofalo et al. 2009; Monzòn-Argüello et al. 2010; Reis et al. 2010; Shamblin et al. 2011b). Genetic structure at regional scales has been detected in the northwestern Atlantic, Brazilian, and Mediterranean nesting aggregations. Among loggerhead turtle rookeries in the southeastern United States, each successive study has discerned additional structure via increased sampling effort. Initial restriction fragment length polymorphism analyses determined that at least two genetic stocks occurred in the southeastern USA, one nesting on

Florida beaches and the other nesting on Georgia and South Carolina beaches (Bowen et al. 1993; Bowen et al. 1994). More extensive sampling and mitochondrial control region sequence analysis added the northwestern Florida rookeries as a distinct management unit (Encalada et al. 1998). Further control region sequence analysis determined that the Dry Tortugas rookery was sufficiently differentiated from proximal mainland beaches to warrant management unit status (Pearce 2001).

The most recent analysis further subdivided peninsular Florida such that a total of 6 management units were proposed for the southeastern USA, corresponding to the beaches from: 1) Virginia through the Ponce Inlet area of northeastern Florida, 2) central eastern Florida, encompassing Volusia County south of Ponce Inlet and Brevard County, 3) southern Florida, represented by peninsular Florida beaches from Juno Beach in southeastern Florida through Keewaydin Island in southwestern Florida, 4) the Dry Tortugas, 5) central western Florida, represented by Sarasota County, and 6) northwestern Florida (Shamblin et al. 2011b). Despite lack of significant differentiation of southernmost sampled rookeries on the Atlantic and Gulf coasts of Florida, the authors speculated that these beaches may host demographically isolated nesting populations based on nesting habitat discontinuity and the relatively large distance separating mainland beaches (Shamblin et al. 2011b).

Although studies have detected structure on the order of 10s of km in some cases, extensive haplotype sharing among loggerhead turtle rookeries occurs at ocean basin and regional scales. The most ubiquitous haplotype in the southeastern USA, CC-A1 (Encalada et al. 1998; Bowen et al. 2005; Shamblin et al. 2011b), is also the dominant haplotype at the Cape Verde rookeries in the eastern Atlantic (Monzòn-Argüello et al. 2010). CC-A2, the second most common haplotype in the southeastern USA and the dominant haplotype at Quintana Roo and Cuban rookeries (Encalada et al. 1998; Bowen et al. 2004; Ruiz U. et al. 2008; Shamblin et al. 2011b) is also the most common haplotype shared among all Mediterranean rookeries analyzed to date (Laurent et al. 1998; Carreras et al. 2007; Garofalo et al. 2009; Chaieb et al. 2010). The two common haplotypes accounted for approximately 79%

(n = 1848) of samples from Atlantic and Mediterranean loggerhead rookeries, with only the Brazilian rookeries lacking any CC-A1 or CC-A2 individuals (Reis et al. 2010). Among southeastern USA rookeries, haplotype CC-A1 was recorded from all beaches sampled, and CC-A2 was found on all peninsular Florida beaches surveyed (Encalada et al. 1998; Bowen et al. 2004; Shamblin et al. 2011b). These two haplotypes accounted for approximately 94% (n = 1201) of samples analyzed from southeastern USA loggerhead rookeries.

Extensive haplotype sharing not only potentially confounds assessments of demographic connectivity among rookeries, overlap of genetic markers among nesting populations also introduces uncertainty into estimates of rookery contributions to mixed aggregations of foraging turtles (Bolker et al. 2007). Analyses of foraging juveniles have demonstrated transport of oceanic stage loggerhead turtle juveniles across entire ocean basin gyres in the North Atlantic, North Pacific, and South Pacific (Bowen et al. 1995; Bolten et al. 1998; Boyle et al. 2009). The presence of an apparently endemic haplotype from Cape Verde among a small sample of juveniles along the Brazilian coast suggests trans-Atlantic dispersal likely also occurs in the South Atlantic (Reis et al. 2009). Extensive analysis of large juveniles and subadults foraging along the continental shelf of the United States indicate that juveniles may home to the vicinity of natal regions to forage, although this structure was much weaker than that inferred among nesting populations in the respective regions (Bowen et al. 2004). As rookery sampling has increased, additional haplotype sharing among regions has been uncovered that could affect interpretations of mixed stock analysis. For example, some portion of CC-A1 turtles foraging in the Mediterranean initially assigned to western Atlantic stocks (eg. Laurent et al. 1998; Carreras et al. 2007) may be attributable to the Cape Verde rookery.

Sequence comparisons of an expanded control region fragment have resulted in the detection of significant novel polymorphism that should improve assessments of hawksbill turtle population structure and mixed stock analyses (Abreu-Grobois et al. 2006). Preliminary utilization of the novel primers described in that study with loggerhead turtles found that 390 bp

CC-A1 was subdivided into four haplotypes with the longer sequences, each being present only in Cape Verde rookeries or in the western Atlantic rookery sampled at Blackbeard Island, Georgia, USA (Monzòn-Argüello et al. 2010). These preliminary data are promising, and baseline sequence data for the expanded mitochondrial control region are required from the major Atlantic rookeries to determine their utility for improved resolution of population structure and mixed stock analyses. We addressed this need through reassessment of population structure among loggerhead turtle rookeries in the southeastern USA using the 817 bp control region fragment. This analysis substantially increased sample sizes compared with previous studies for most sampled populations and added novel haplotype data from the Cay Sal Bank rookery, the most significant nesting population in the Bahamas (Dow et al. 2007).

Methods

Field Methods

Samples from 2260 individual loggerhead turtles or nests were collected from 12 southeastern USA beach locations and Cay Sal Bank, Bahamas from the 1999 through 2010 nesting seasons (Table 3.1). DRT samples were those analyzed in (Bowen et al. 2004). The 2002-2005 CSB and 2006-collected CAP, CAN, MEL, JUN, FTL, KEY, CSK, SGI, and SJP samples were those analyzed in Shamblin et al. (2011b). Sample sites were chosen to represent the extent of loggerhead turtle nesting in the USA where nesting densities were sufficient to provide adequate sample sizes and the Bahamian beach with the highest nesting density (Fig 3.1). Most rookeries are represented by skin samples that were collected from the shoulder region or rear flippers of nesting females using 6-mm biopsy punches following oviposition and during the nest covering and camouflaging process. Precautions were taken to ensure that each nesting female was represented only once, either via tagging to prevent duplicate sampling, or by microsatellite genotyping which permitted recognition of individual turtles (Shamblin et al. 2007; Shamblin et al. 2009; 15 loci, minimum non-exclusion probability of identity of

2.07 X 10^{-24}, microsatellite data not shown). OSS samples were eggshells collected within 12 hours of oviposition and genotyped at 15 or 17 loci to assign individual identity (DNA extraction and sample assignment methods detailed in Shamblin et al. 2011a). Nest samples were dead hatchlings or hatched eggshells collected during post-emergence nest evaluations, and each nest was represented by a single sample. Sampled clutches from 2006 were laid June 15 through June 24. A ten-day sampling window was chosen to maximize sample sizes while minimizing the probability of re-sampling females. The average inter-nesting interval for southeastern USA loggerhead turtles is approximately 14 days, with females rarely re-nesting at fewer than 11 days (reviewed in Dodd 1988). Because FTL samples were comprised of tagged females and nest samples from unknown females, all were genotyped and analyzed for parentage at a minimum of 6 loci to remove duplicate samples from individual females. Samples were stored in 70% ethanol, 95% ethanol, or saturated NaCl-DMSO buffer prior to DNA extraction.

Laboratory Methods

Genomic DNA was extracted using the DNeasy blood and tissue kit (QIAGEN) following standard protocols. Polymerase chain reaction (PCR) amplifications of an 817 bp fragment of the mitochondrial control region were carried out using primers LCM15382 and H950g (Abreu-Grobois et al. 2006). PCR reactions were carried out in 20 μl volumes containing 10mM Tris, pH 8.4; 50 mM KCl, 0.5 μM of each primer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 unit of Taq DNA Polymerase, and approximately 10 to 30 ng of genomic DNA. PCR cycling parameters were as follows: 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 60 seconds, 72°C for 30 seconds; and a final extension of 72°C for 10 minutes. PCR products were purified by adding 2 μl of ExoSAP-IT (USB Corporation) to 7 μl of PCR amplicon and incubated according to manufacturer's instructions. The mtDNA amplicons were sequenced using ABI BigDye v3.1 (PE Applied Biosystems) and an ABI 3730*xl* DNA Analyzer with LCM15382 and an internal sequencing primer CC443, TGATCTATTCTGGCCTCTG. Neg-

ative controls were included in each batch of PCR amplifications and sequencing reactions to detect contamination.

Data Analysis

Sequences were aligned, edited, and compared to previously described haplotypes using the program Sequencher 4.2 (Gene Codes Corporation). Sequences were assigned haplotype designations after nomenclature published on the Archie Carr Center for Sea Turtle Research (ACCSTR) website (http://accstr.ufl.edu/cclongmtdna.html). Original, short haplotypes received consecutive number designations based on the 390-bp sequence. Haplotypes based on the 817 bp fragment retain their original designations and receive additional numeral suffixes to account for any novel polymorphisms detected within the expanded sequences. Samples producing novel or ambiguous sequences were subjected to a second round of DNA extraction, PCR amplification, and sequencing for verification. Novel haplotypes were deposited with Genbank and ACCSTR.

Pairwise F_{ST} comparisons, pairwise exact tests of population differentiation, and analysis of molecular variance (AMOVA) were conducted using the software Arlequin version 3.1 (Excoffier et al. 2005). Significance values for AMOVA were obtained from 10,000 permutations. Tests of temporal variation of haplotype frequencies were conducted using AMOVA. All interannual samples for each site that were not significantly different were pooled for spatial analyses. Spatial structure was examined using AMOVA, pairwise F_{ST} comparisons, and exact tests of population differentiation. Exact tests of population differentiation were conducted with 100,000 permutations and 10,000 dememorization steps after the method of Raymond and Rousset (1995).

Following pairwise F_{ST} comparisons and exact tests of population differentiation, all proximal sample sites that were not significantly different were pooled for further analyses. Following rookery clustering for management unit tests, haplotype frequencies from proposed southeastern United States management units were compared with published data from

other North Atlantic loggerhead rookeries in two sets of tests. The first analysis consisted of comparisons based on 390 bp haplotypes only, and included published haplotype data from Cape Verde (n = 186; Monzòn-Argüello et al. 2009); Quintana Roo, Mexico (Encalada et al. 1998); and southwestern Cuba (Ruiz U. et al. 2008). The second analysis consisted of comparisons of proposed southeastern US management units with a smaller Cape Verde data set (n = 128, Monzòn-Argüello et al. 2010) based on 390 and 817 bp fragments to quantify the utility of the expanded control region sequence in differentiating rookeries. Significance of the final round of pairwise F_{ST} comparisons and exact tests of population differentiation were adjusted using sequential Bonferroni correction with a table-wide $\alpha = 0.05$ (Rice 1989).

Results

Haplotype diversity

Sequence alignments of the 390 bp control region fragment revealed 33 polymorphic sites, corresponding to 25 transitions and 9 indels (Table 3.2). Position 358 contained both an indel and a transition. Eighteen of the polymorphic sites differed only between the two lineages previously described based on partial control region sequence analysis (Encalada et al. 1998). Polymorphic sites defined 18 haplotypes, 13 of which were previously described from southeastern USA rookeries (Encalada et al. 1998; Bowen et al. 2004; Shamblin et al. 2011). Haplotype CC-A21, formerly recovered from two foraging juveniles around the Straits of Gibraltar (Revelles et al. 2007), was found in four nesting females from CSK in the present study. Haplotype CC-A27, described from a juvenile foraging in the vicinity of the Gimnesies Islands in the western Mediterranean (Carreras et al. 2006), was recovered from an individual nesting at CSB in northwestern Florida. The remaining haplotypes were novel: CC-A36, CC-A41, and CC-A51.

Sequence comparisons of the 817 bp fragment revealed 24 additional polymorphic positions, corresponding to 22 transitions, one transversion, and one indel (Table 3.2). Fourteen of the sites varied only between the two lineages. Novel variable positions subdivided five 390

bp haplotypes into 12 variants for total of 28 haplotypes resolved with the expanded control region fragment. Short haplotype CC-A1 was subdivided into four subhaplotypes: CC-A1.1, CC-A1.2, CC-A1.3, and CC-A1.4. Haplotypes CC-A1.3 and CC-A1.4 were variants previously described from Cape Verde rookeries (Monzòn-Argüello et al. 2010) and were found at low frequency in several Florida rookeries. CC-A1.1, formerly described from a Georgia rookery (Monzòn-Argüello et al. 2010), was found at each USA rookery sampled and was absent only from Cay Sal Bank, Bahamas. Short haplotype CC-A2 was also subdivided into five variants: CC-A2.1, CC-A2.3, CC-A2.4, CC-A2.5, CC-A2.N. Haplotype CC-A2.1, previously found at the rookeries of Cape Verde and Calabria, Italy (Garofalo et al. 2009; Monzòn-Argüello et al. 2010), was detected in all sampled rookeries except CAP, OSS, and SJP.

Interannual variation and regional population structure

Among AMOVA tests of interannual haplotype frequency differentiation, only SAL yielded a significant difference between sample years (F_{ST} = 0.1224, p = 0.014; Table 3.3). The sample size for each year was relatively small, particularly for 1999 (n = 6), and sampling error may have contributed to apparent temporal structuring. Because data were limited for this rookery, we combined annual sample sets for spatial analyses.

The two most common haplotypes, CC-A1.1 and CC-A2.1, accounted for approximately 85% of all individuals sampled, but these haplotypes exhibited strong partitioning among sample sites (Table 3.4). Of 78 pairwise comparisons based on the 390 bp haplotypes, 63 pairwise F_{ST} comparisons and 63 exact tests of population differentiation were significant. Among 817 bp haplotype pairwise comparisons, 67 pairwise F_{ST} comparisons and 60 exact tests of population differentiation were significant. Comparisons involving proximal sample sites accounted for most of the nonsignficant differences (Table 3.5). Sites for which both F_{ST} comparisons and exact tests of population differentiation yielded no differences were pooled for a second round of pairwise comparisons (CAP and OSS; CAN and MEL; JUN and FTL;

SAL and DRT; SGI, CSB, and SJP). The seven resulting regional clusters following sample site pooling were 1) South Carolina and Georgia, 2) central eastern Florida, 3) southeastern Florida, 4) Cay Sal and Dry Tortugas, 5) southwestern Florida, 6) central western Florida, and 7) northwestern Florida. Pairwise comparisons among the seven regional groupings were all significant after sequential Bonferroni correction for multiple tests.

Southeastern USA management units in a North Atlantic context

With 390 bp haplotypes for all North Atlantic rookeries considered, the overall structure among North Atlantic loggerhead turtle management units was strong (F_{ST} = 0.190, p < 0.00001). Quintana Roo, southwestern Cuba, all proposed southeastern USA management units, and Cape Verde rookeries were all significantly different in pairwise F_{ST} comparisons and exact tests of population differentiation following sequential Bonferroni correction for multiple tests except SEFL and Cuba (F_{ST} = 0.036, p = 0.034; Table 3.6). While Cape Verde was significantly different from all Northwest Atlantic rookeries in 390 bp haplotype frequency comparisons, use of the expanded 817 bp haplotypes markedly increased pairwise F_{ST} values for southeastern USA management unit and Cape Verde comparisons (Table 3.7).

Discussion

Population structure

Larger sample sizes collected across multiple nesting seasons provided additional support for the latitudinal genetic break across central Florida inferred from previous analysis (Shamblin et al. 2011b). Haplotype frequency transitions were detected between central and southern sample sites on both coasts of Florida. Moreover, the expanded dataset indicated that southernmost sampled rookeries along the Gulf and Atlantic coasts of the Florida were genetically distinct, a relationship which remained ambiguous following the previous analysis. Shamblin et al. (2011b) speculated that these rookeries were likely demographically isolated given the discontinuity of available nesting habitat around the southern tip of the Florida peninsula

and the rarity of recorded exchange of nesting females among west coast and east coast Florida rookeries. Genetic data now corroborate the hypothesis of demographic partitioning between Florida's Gulf and Atlantic coasts.

Lack of haplotype frequency differentiation between Cay Sal, Bahamas and Dry Tortugas rookeries may reflect historical genetic signature rather than contemporary demographic connectivity. Both rookeries were dominated by haplotype CC-A2.1 (86% of SAL and 90% of DRT), potentially limiting the power to distinguish them. Haplotype frequencies for DRT were significantly different from KEY (F_{ST} = 0.309, p < 0.00001), the closest mainland rookery analyzed approximately 200 km northeast of DRT. DRT and SAL are separated by 270 km, suggesting that these insular rookeries may not be connected through demographically relevant levels of non-natal female exchange. Future research should reevaluate the relationship of these rookeries through analysis of larger sample sizes, deeper mitogenomic sequencing, and demographic studies.

Use of expanded control region haplotypes based on longer sequences did not significantly improve the resolution of population structure among southeastern USA rookeries in most comparisons. The 817 bp CC-A1 and CC-A2 sequences were each dominated by a single common haplotype that was widely distributed. The exception to this pattern involved the southeastern Florida rookeries compared to all others in the southeastern USA. Haplotype CC-A1.3 accounted for a relatively large proportion of the CC-A1 turtles sampled in southeastern Florida (31%), whereas this haplotype accounted for only 0.4% and 0.7% of the CC-A1 turtles nesting in central eastern and central western (CSK) Florida, respectively and was not detected in southwest Florida. Southeastern Florida rookeries were considered distinct prior to use of the expanded control region fragments (Shamblin et al. 2011b). This suggests that analyses based on deeper mitogenomic sequencing may reinforce inferences of population subdivision among currently proposed management units in the region rather than uncovering any additional cryptic structure among rookeries within them. Polymorphism detected in the larger fragment did increase differentiation between the southeastern

USA and Cape Verde nesting aggregations, as previously demonstrated (Monzòn-Argüello et al. 2010). Therefore continued use of the longer sequences is justified for defining rookeries in a broader context, even if they do not substantially improve resolution of regional assessments.

Phylogeography

Preliminary restriction fragment length polymorphism analysis of the mitochondrial genome detected two lineages of loggerhead turtles globally, each represented by rookeries in the Indo-Pacific and Atlantic (Bowen et al. 1994). Bowen et al. (1994) hypothesized that one lineage may have evolved in the Atlantic (represented in the present study by CC-A1 and its related haplotypes), with a precursor to haplotype F (found at the Oman rookery in the Indian Ocean) invading that basin during an interglacial period around the southern tip of Africa. A loggerhead injured while foraging in Kuwait was found to carry haplotype CC-A11 and was considered to be of Atlantic origin because this haplotype had previously been recovered from turtles foraging in the Atlantic (Al-Mohanna and George 2010). However, given that the largest nesting population of loggerhead turtles in the Indo-Pacific, if not the world, occurs in Oman (Baldwin et al. 2003), it seems more probable that this turtle represents the proximate Omani rookery. Haplotype CC-A11 has been recorded at low frequency in four Atlantic rookeries represented by three different expanded control region variants (Monzòn-Argüello et al. 2010; Nielsen 2010; present study), and is intermediate in the parsimony network between the clade containing CC-A2 and the haplotype CC-A1 variants. These data suggest evolutionarily recent connectivity between the rookery in Oman and those of the North Atlantic.

Refinements of phylogeographic inferences must be tempered by the limited novel polymorphism detected in the expanded control region sequences. However, the few variable positions recovered do provide sufficient resolution to propose a plausible colonization scenario for the CC-A1 lineage in the Atlantic. Cape Verde is the most equatorial of the large

loggerhead turtle nesting aggregations in the Atlantic harboring CC-A1. Given the derived position of CC-A1.1 relative to CC-A1.3 and CC-A1.4 in the haplotype network and the presence of CC-A1.3 and CC-A1.4 at both Cape Verde and western Atlantic rookeries, Cape Verde may have served as a stepping-stone for the CC-A1 lineage that ultimately colonized the southeastern USA. Thus CC-A1.1 from southeastern USA rookeries, the most common variant thus far described from the Atlantic with respect to numbers of nesting turtles, is also likely the youngest major lineage representing CC-A1. Short haplotype CC-A1 has also been reported at low frequency from Cuban rookeries (Ruiz U. et al. 2008). Expanded sequences are unavailable for the southwest Cuban rookeries, so these rookeries cannot be excluded as a proximate source for the CC-A1 lineage that has colonized the entire southeastern USA coastline.

Encalada et al. (1998) hypothesized that the Mediterranean was colonized by the CC-A2 lineage following the Wisconsin glaciation. Haplotype CC-A2.1 was the most common variant of CC-A2 detected in the present study and the only variant recorded for Calabria, Italy rookeries (Garofalo et al. 2009). Therefore the expanded control region sequences were less informative for CC-A2 than CC-A1 and did not permit any elaborations of phylogeographic scenarios previously proposed. Since most CC-A2 turtles nesting at Northwest Atlantic rookeries and the only Mediterranean rookery for which expanded CC-A2 haplotypes were available for comparison could not be distinguished, this may reflect a broader pattern across the Mediterranean rookeries. Deeper sequencing of the mitogenome is warranted to determine whether any diagnostic variable positions occur that will distinguish turtles of western Atlantic and Mediterranean origin. The presence of CC-A2 at high frequency among nearly all western Atlantic rookeries examined (Encalada et al. 1998; Bowen et al. 2004; Ruiz U. et al. 2008; Shamblin et al. 2011b) as well as all Mediterranean rookeries surveyed (Encalada et al. 1998; Carreras et al. 2006; Garofalo et al. 2009; Chaieb et al 2010) demonstrates that although natal philopatry is the paradigm, this species is capable of long distance nesting relocations (Encalada et al. 1998).

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Table 3.1: Sample collection data and nesting data for sampled rookeries in the present study. Nests are annual mean nest counts from 2002-2006. Nesting data were from South Carolina Department of Natural Resources, Georgia Department of Natural Resources, and Florida Fish and Wildlife Research Institute's Statewide Nesting Beach Survey, Florida Fish and Wildlife Conservation Commission.

Code	Sample Beach	Nests	Years Sampled	Sample Size	Sample Type
CAP	Cape Island, South Carolina, USA	735	2006	52	F[a]
OSS	Ossabaw Island, Georgia, USA	165	2008, 2009	89	E[b]
CAN	Canaveral National Seashore, Florida, USA	2738	2001, 2003, 2004, 2006	314	F
MEL	Melbourne Beach, Florida, USA	9439	2003, 2004, 2006-2008	750	F
JUN	Juno Beach, Florida, USA	3136	2003, 2004, 2006	141	F
FTL	Ft. Lauderdale, Florida, USA	575	2003, 2006	75	F + N[c]
SAL	Cay Sal Bank, Bahamas	100-500[d]	1999, 2000	21	F
DRT	Dry Tortugas, Florida, USA	151[e]	1995-1999[f]	32	N
KEY	Keewaydin Island, Florida, USA	128	2004, 2006-2010	208	F
CSK	Casey Key, Florida, USA	449	2006-2010	463	F
SGI	St. George Island, Florida, USA	106	2006	13	N
CSB	Cape San Blas, Florida, USA	36	2002-2005; 2010	87	F
SJP	St. Joe Peninsula State Park, Florida, USA	92	2006	15	N

[a]Female samples were skin biopsies obtained from nesting turtles during nest covering and camouflaging

[b]Egg samples were egg shells collected within 12 hours of oviposition

[c]Nest samples were dead hatchlings or embryos collected during post emergence nest evaluations

[d]Bahamas data from Dow et al. 2007, numbers represent total crawls, not just nests

[e]Nest counts available from 2003 and 2004 only

[f]Precise sampling year unknown, but falls within the range presented in Pearce (2001)

Table 3.2: Variable positions for the 817 base pair control region sequence for southeastern USA loggerhead turtle rookery haplotypes. Thirty-two polymorphic sites that varied only between CC-A1 and CC-A2 lineages were excluded for legibility. Shading corresponds to variable sites in the 390 bp haplotypes.

	Polymorphic Positions (base pairs)																								
390 bp			32	51	78	79	96	119	136	161	162	188	210	244	312	358	363								
817 bp	73	171	204	223	250	251	268	291	308	333	334	360	382	416	484	530	535	555	561	562	572	658	681	701	800
CC-A2.1	A	A	C	C	G	T	A	A	C	T	A	G	T	A	A	A	A	C	C	C	G	A	C	A	C
CC-A2.3	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
CC-A2.4	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
CC-A2.N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
CC-A3.1	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A5.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
CC-A7.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	.
CC-A7.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
CC-A8.1	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A9.1	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A10.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	T	.	.	.	.
CC-A10.3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	-
CC-A13.1	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A20.1	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A36.1	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.
CC-A36.2	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	T	.	T	.	.	.	.
CC-A43.1	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A51.1	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
CC-A1.1	.	.	T	T	.	.	.	.	.	C	G	.	C	G	.	-	.	.	.	.	.	G	.	G	.
CC-A1.2	.	.	T	T	.	.	.	.	.	C	G	.	C	G	.	-	.	.	.	.	.	.	.	G	.
CC-A1.3	.	.	T	T	.	.	.	.	.	C	G	.	C	G	.	-	.	.	.	T	.	G	.	.	.
CC-A1.4	.	.	T	T	.	.	.	.	.	C	G	.	C	G	.	-	.	.	.	T	.	G	.	G	.
CC-A11.3	.	.	T	T	.	.	.	.	.	C	.	.	C	G	.	-	.	T	.	.	.	G	.	G	.
CC-A14.1	.	.	T	T	A	.	.	.	.	C	.	.	C	.	.	-	.	.	.	T	.	G	.	.	.
CC-A21.1	.	.	T	T	.	C	.	.	.	C	G	.	C	G	.	-	.	.	.	.	.	G	.	G	.
CC-A27.1	.	.	T	T	.	.	.	.	T	C	G	.	C	G	.	-	.	.	.	.	.	G	.	G	.
CC-A41.1	.	.	T	T	.	.	.	.	.	C	G	.	.	G	.	-	.	.	.	.	.	G	.	G	.

Table 3.3: Expanded control region haplotype frequencies for loggerhead turtles in the North Atlantic. Sample site abbreviations for rookeries sampled in this study are explained in Table 3.1.

Haplotype	CAP	OSS	CAN	MEL	JUN	FTL	SAL	DRT	KEY	CSK	SGI	CSB	SJP	CV[a]
CC-A1.1	52	89	210	458	20	7		1	106	250	11	73	12	
CC-A1.2			3	12	1					10				
CC-A1.3				3	8	8		1		2				79
CC-A1.4			8	12	6	1	1			7		2		6
CC-A1.5														3
CC-A2.1			78	197	76	47	18	28	80	108	1	9		2
CC-A2.3				1	3					2				
CC-A2.4				1	2	6				3				
CC-A2.5				1	1					3				
CC-A2.N										1				
CC-A3.1			7	30	10	4	1		13	47	1	1		
CC-A5.1				2										
CC-A7.1			2	5	2					6			1	
CC-A7.2				2										
CC-A8.1										1				
CC-A9.1			1	1	2			2	1	1		1		
CC-A10.1			1	8	5	2				8			1	
CC-A10.3				1										
CC-A11.2														1
CC-A11.3							1			1				
CC-A13.1				1	1					2				
CC-A14.1			3	12	1				1	5			1	
CC-A17.1														27
CC-A17.2														6
CC-A20.1				2	2				5	1				
CC-A21.1										4				
CC-A27.1												1		
CC-A36.1				1										
CC-A36.2										1				
CC-A41.1			1											
CC-A43.1					1									
CC-A47.1														1
CC-A51.1									2					

[a]Cape Verde, haplotype data from Monzón-Argüello et al. 2010.

Table 3.4: F_{ST} values from AMOVA temporal tests of expanded control region haplotypes. Sample site abbreviations for rookeries sampled in this study are explained in Table 3.1.

	F_{ST}	P value
CAN	0.0015	0.343
MEL	0.0050	0.082
JUN	0.0062	0.220
FTL	-0.0092	0.592
SAL	0.1224	0.014
KEY	0.0167	0.118
CSK	0.0035	0.193
CSB	-0.0103	0.548

Table 3.5: Pairwise F_{ST} values for southeastern USA rookery haplotype frequency comparisons. Comparisons based on 390 bp haplotypes are above the diagonal. Comparisons based on the 817 bp haplotypes are below the diagonal. Rookery abbreviations are explained in Table 3.1. * indicates significant comparisons $\alpha = 0.05$ without correction for multiple tests.

	CAP	OSS	CAN	MEL	JUN	FTL	SAL	DRT	KEY	CSK	SGI	CSB	SJP
CAP		0.000	0.156*	0.165*	0.510*	0.672*	0.915*	0.925*	0.287*	0.184*	0.211	0.075*	0.225*
OSS	0.000		0.173*	0.174*	0.556*	0.723*	0.942*	0.947*	0.318*	0.198*	0.314*	0.100*	0.326*
CAN	0.162*	0.179*		0.002	0.246*	0.333*	0.475*	0.495*	0.052*	0.018*	0.019	0.048*	0.055
MEL	0.169*	0.179*	0.002		0.198*	0.271*	0.407*	0.425*	0.029*	0.006*	0.036	0.066*	0.065
JUN	0.512*	0.557*	0.252*	0.209*		0.005	0.080*	0.105*	0.080*	0.155*	0.333*	0.396*	0.351*
FTL	0.639*	0.697*	0.323*	0.269*	0.004		0.038	0.060*	0.141*	0.226*	0.466*	0.530*	0.480*
SAL	0.919*	0.945*	0.455*	0.393*	0.074*	0.052*		-0.022	0.270*	0.355*	0.686*	0.707*	0.672*
DRT	0.927*	0.949*	0.469*	0.403*	0.097*	0.074*	-0.013		0.296*	0.376*	0.747*	0.705*	0.733*
KEY	0.287*	0.318*	0.039*	0.021*	0.115*	0.170*	0.292*	0.309*		0.018*	0.123*	0.169*	0.153*
CSK	0.186*	0.200*	0.018*	0.007*	0.164*	0.221*	0.346*	0.361*	0.180*		0.047	0.088*	0.072*
SGI	0.211*	0.314*	0.026	0.043	0.342*	0.435*	0.699*	0.753*	0.124*	0.052		-0.035	-0.032
CSB	0.081*	0.108*	0.047*	0.064*	0.393*	0.497*	0.689*	0.710*	0.155*	0.084*	-0.035		0.014
SJP	0.225*	0.326*	0.056	0.067*	0.352*	0.444*	0.685*	0.740*	0.153*	0.072*	-0.032	0.005	

Table 3.6: Pairwise F_{ST} values for comparisons among proposed management units for southeastern USA, Quintana Roo, Cuba, and Cape Verde loggerhead turtle rookeries. Comparisons based on 390 bp haplotypes are above the diagonal. Comparisons based on the 817 bp haplotypes are below the diagonal. * indicates significance at $\alpha = 0.05$ prior to correction for multiple tests. ** indicates significance after sequential Bonferroni correction with a table-wide $\alpha = 0.05$. NRTH is northern management unit sites. CEFL is central eastern Florida (CAN and MEL). SEFL is southeastern Florida (JUN and FTL), DTSL is DRT and SAL. SWCB is southwestern Cuba. QRMX is Quintana Roo. SWFL is southwestern Florida (KEY). CWFL is central western Florida (CSK). NWFL is northwestern Florida SGI, CSB, SJP). CPVD is Cape Verde.

	NRTH	CEFL	SEFL	DRSL	SWCB	QRMX	SWFL	CWFL	NWFL	CPVD
NRTH		0.172**	0.598**	0.916**	0.860**	0.898**	0.356**	0.214**	0.105**	0.258**
CEFL	0.177**		0.242**	0.445**	0.344**	0.365**	0.036**	0.010**	0.062**	0.132**
SEFL	0.586**	0.244**		0.093**	0.036*	0.071**	0.103**	0.183**	0.427**	0.403**
DRSL	0.937**	0.419**	0.084**		0.093**	0.209**	0.316**	0.386**	0.719**	0.621**
SWCB	NA	NA	NA	NA		0.055	0.191**	0.271**	0.588**	0.493**
QRMX	NA	NA	NA	NA	NA		0.213**	0.277**	0.606**	0.491**
SWFL	0.356**	0.026**	0.132**	0.318**	NA	NA		0.018**	0.175**	0.197**
CWFL	0.217**	0.010**	0.184**	0.364**	NA	NA	0.018**		0.089**	0.128**
NWFL	0.107**	0.066**	0.421**	0.712**	NA	NA	0.170**	0.092**		0.128**
CPVD	0.728**	0.451**	0.356**	0.555**	NA	NA	0.418**	0.382**	0.568**	

Table 3.7: Pairwise F_{ST} values for proposed management units for southeastern USA and Cape Verde loggerhead turtle rookeries. Comparisons based on 390 bp haplotypes are above the diagonal. Comparisons based on the 817 bp haplotypes are below the diagonal. All comparisons were significant following sequential Bonferronni correction with table-wide α = 0.05. NRTH is northern management unit sites. CEFL is central eastern Florida (CAN and MEL). SEFL is southeastern Florida (JUN and FTL), DTSL is DRT and SAL. SWFL is southwestern Florida (KEY). CWFL is central western Florida (CSK). NWFL is northwestern Florida SGI, CSB, SJP). CPVD is Cape Verde.

	NRTH	CEFL	SEFL	DRSL	SWFL	CWFL	NWFL	CPVD
NRTH		0.172	0.598	0.913	0.356	0.214	0.100	0.273
CEFL	0.177		0.242	0.411	0.036	0.010	0.066	0.118
SEFL	0.586	0.244		0.060	0.103	0.183	0.436	0.397
DRSL	0.937	0.419	0.084		0.267	0.347	0.695	0.594
SWFL	0.356	0.026	0.132	0.318		0.018	0.183	0.184
CWFL	0.217	0.010	0.184	0.364	0.018		0.094	0.116
NWFL	0.110	0.064	0.420	0.712	0.168	0.091		0.120
CPVD	0.736	0.454	0.360	0.562	0.423	0.386	0.577	

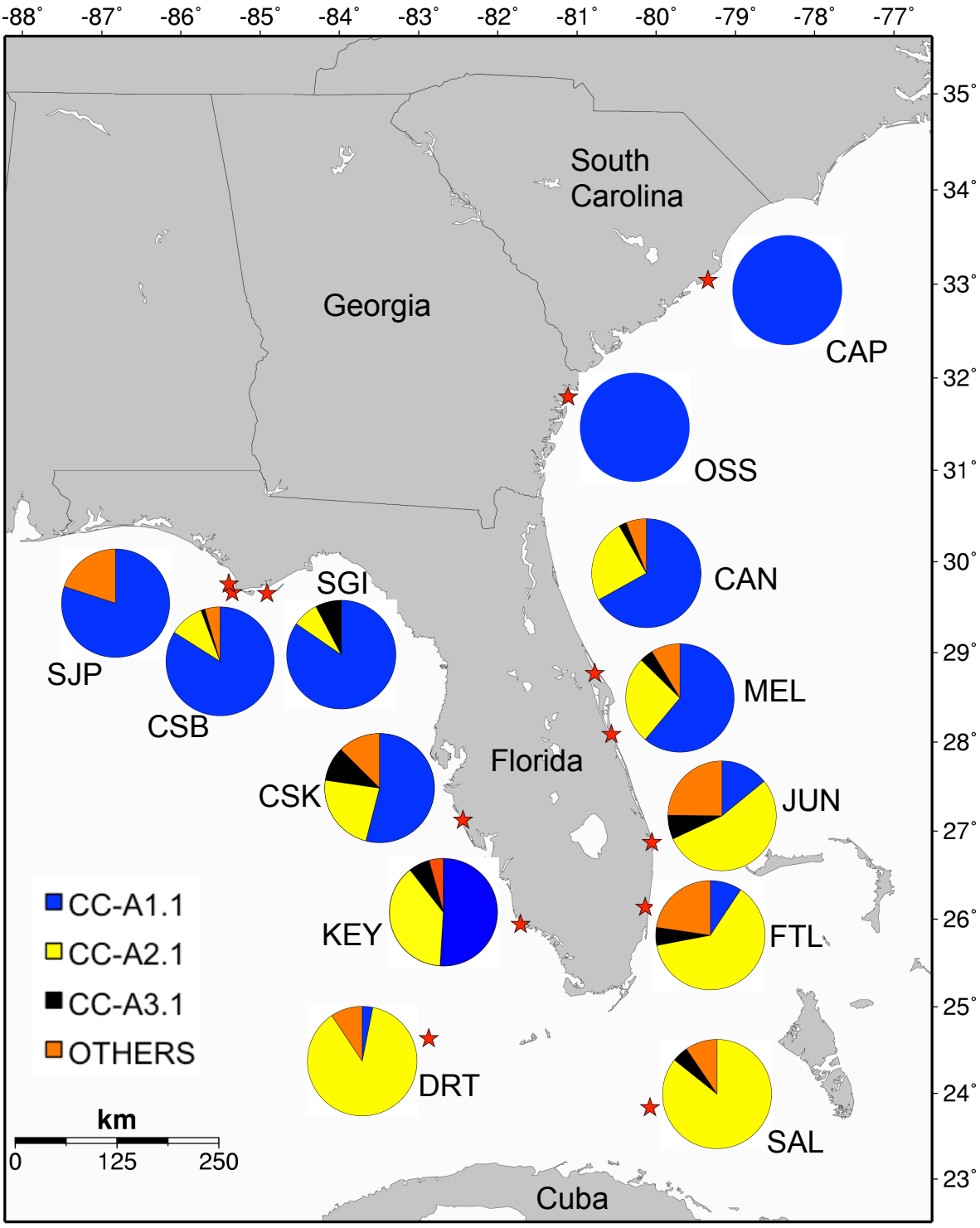

Figure 3.1: Sample site locations and frequency distributions of common control region haplotypes of southeastern USA and Cay Sal, Bahamas loggerhead rookeries.

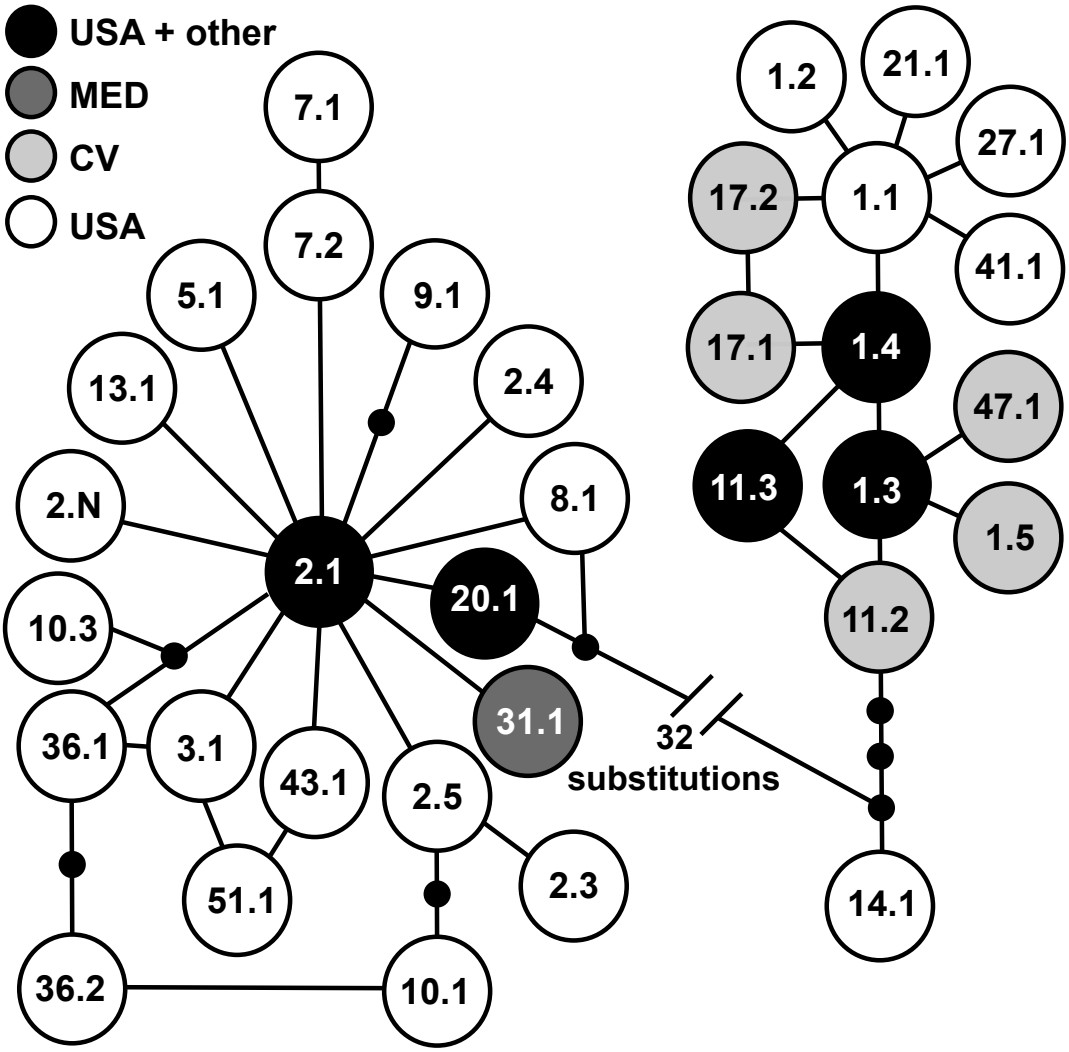

Figure 3.2: Unrooted parsimony network of expanded control region haplotypes illustrating the two deep lineages present in the Atlantic and Mediterranean basins. CV is Cape Verde, data from Monzòn-Argüello et al. (2010). MED is Mediterranean, data from Calabria, Italy (Garofalo et al. 2009).

Chapter 4

Population structure of green turtle rookeries in the southern Greater Caribbean revisited: inferences from mitogenomic sequences[1]

[1]Shamblin BM, Bjorndal KA, Bolten AB, Hillis-Starr ZM, Lundgren I, Naro-Maciel E, Nairn CJ. To be submitted to Molecular Ecology.

Abstract

Analyses of mitochondrial control region polymorphism have supported the presence of several demographically independent green turtle rookeries globally and in the Greater Caribbean region. However, extensive sharing of common haplotypes based on 490-bp control region sequences confounds assessment of the scale of natal homing and population structure among regional rookeries, particularly those of Aves Island and Suriname. To determine whether informative variation occurred outside of the established control region fragment, we screened the majority of the mitochondrial genome of several green turtles carrying haplotype CM-A5 and representing the rookeries of Buck Island, United States Virgin Islands (USVI); Aves Island, Venezuela; Galibi, Suriname; and Tortuguero, Costa Rica. We identified five single nucleotide polymorphisms (SNPs) that subdivided CM-A5 among regions. Significant haplotype frequency differentiation supports demographic independence of Aves Island and Suriname, highlighting the need to manage the smaller Aves rookery as a distinct management unit. Aves Island and Buck Island rookeries shared mitogenomic haplotypes; however frequency divergence suggests that the Buck Island rookery is sufficiently demographically isolated to warrant management unit status. Given that widespread haplotype sharing is common among rookeries in most marine turtle species, mitogenomic sequencing may elucidate inferences of population structure and phylogeography, as well as improve the resolution of mixed stock analyses.

Introduction

Defining population structure of highly vagile marine species can be challenging given their ability to disperse over vast spatial scales. For marine turtles, population boundaries are delimited on the basis of female philopatry to natal rookeries (Bowen et al. 1992; Bowen et al. 1993a; Norman et al. 1994). It is therefore critical from a conservation perspective to properly characterize the scale of this natal homing behavior to ensure that demographically

isolated rookeries receive adequate recognition and protection. The complex life histories of green turtles (*Chelonia mydas*), loggerhead turtles (*Caretta caretta*), hawksbill turtles (*Eretmochelys imbricata*), and Kemp's ridley turtles (*Lepidochelys kempii*) are similar with respect to developmental and seasonal migrations (Musick and Limpus 1997). The first step of the life cycle involves an oceanic juvenile dispersal stage that sometimes encompasses entire ocean basin gyres (Bowen et al. 1995; Bolten et al. 1998; Monzòn-Argüello et al. 2010a). Following the oceanic stage, turtles often recruit to neritic areas where they shift to benthic foraging (Musick and Limpus 1997). Juvenile and adult foraging aggregations are often comprised of individuals from multiple nesting populations (reviewed in Bowen and Karl 2007). As these foraging grounds are often not proximal to nesting beaches, adult turtles make seasonal shuttling migrations between foraging grounds and breeding grounds adjacent to nesting beaches during their reproductive years (Carr et al. 1978). Characterizing the migratory connectivity of source rookeries and foraging aggregations is an important conservation consideration (Harrison and Bjorndal 2006), given the need to protect highly migratory species throughout their life cycle (Martin et al. 2007).

Genetic tools have proven invaluable in delimiting marine turtle nesting populations and estimating rookery contributions to juvenile and adult foraging aggregations (Bowen and Karl 2007). Analyses of mitochondrial DNA (mtDNA) polymorphisms at global, ocean basin, and regional scales have provided strong evidence for regional natal homing by female green turtles (Meylan et al. 1990; Bowen et al. 1992; Norman et al. 1994; Encalada et al. 1996; Bjorndal et al. 2006; Dethmers et al. 2006; Formia et al. 2006; Bourjea et al. 2007). A study of three Australian green turtle genetic stocks also confirmed that males are philopatric to breeding grounds in the vicinity of their natal regions, despite overlap of the stocks on foraging grounds (FitzSimmons et al. 1997a). However, the precise scale of this natal neighborhood remains unresolved and may vary in different regions in addition to among species. Most green turtle rookeries separated by 500 km or more have significantly

different haplotype frequencies, whereas many comparisons at finer scales have failed to detect significant differentiation (reviewed in Bowen and Karl 2007).

Although female natal philopatry appears to be the primary force shaping population structure of marine turtle rookeries within ocean basins (Bowen et al. 1992), this homing behavior must occasionally break down to permit colonization of novel nesting habitats (Carr et al. 1978). Combined with slow evolution of the mitochondrial genome of marine turtles relative to many other vertebrates (Avise et al. 1992; Bowen et al. 1993b), this ability to colonize sites distant from their natal regions has lead to extensive haplotype sharing (based on 380 bp to 500 bp control region sequences) among rookeries over large spatial scales in multiple species. In the western Atlantic, haplotype A was recorded from hawksbill turtles rookeries in Puerto Rico, United States Virgin Islands, Barbados, and Brazil; haplotype F was recovered from hawksbill rookeries in Belize, Puerto Rico, and the United States Virgin Islands (Bass et al. 1996). Haplotype CC-A1 is the dominant haplotype in loggerhead turtle rookeries in the southeastern USA and Cape Verde in the eastern Atlantic (Encalada et al. 1998; Bowen et al. 2004; Monzòn-Argüello et al. 2009); CC-A2 is the dominant haplotype of the Mexican and Cuban loggerhead turtle rookeries, is the second most common haplotype in the southeastern USA, and dominates the haplotype profile of every characterized rookery in the Meditteranean basin (Encalada et al. 1998; Bowen et al. 2004; Carreras et al. 2007; Ruiz U. et al. 2008; Garofalo et al. 2009; Chaieb et al. 2010). Haplotype A was recorded from all Atlantic, Indian, and western Pacific Ocean leatherback turtle rookeries sampled, being absent only in the eastern Pacific stocks (Dutton et al. 1999).

Among green turtle rookeries, CM-A8 is the most common haplotype in the southern Mozambique channel rookeries in the southwest Indian Ocean and among all of the insular rookeries surveyed in the equatorial Atlantic from coastal Africa to Ascension Island and the Brazilian islands of Trindade and Atol das Rocas (Figure 4.1; Encalada et al. 1996; Bjorndal et al. 2006; Formia et al. 2006; Bourjea et al. 2007; Formia et al. 2007). Rookeries in the southwest Indian Ocean also share haplotypes with rookeries from Malaysia to the Great

Barrier Reef to Micronesia, thousands of kilometers away (A1, A2, and C3; Dethmers et al. 2006; Bourjea et al. 2007). In the Greater Caribbean region, haplotypes CM-A1, CM-A3, and CM-A5 account for approximately 95% of green turtles analyzed at rookeries (n = 550; Encalada et al. 1996; Bjorndal et al. 2005; Ruiz-Urquiola et al. 2010) and 86% of turtles analyzed at foraging grounds from Barbados to North Carolina (n = 937; Bass et al. 1998; Lahanas et al. 1998; Bass and Witzell 2000; Bagley 2003; Luke et al. 2004; Bass et al. 2006; Bjorndal and Bolten 2008b). These haplotypes are shared among several rookeries: CM-A1 in Mexico, Cuba, and Florida; CM-A3 in Mexico, Cuba, Florida, Costa Rica, Suriname, and Aves Island; CM-A5 is the most common haplotype at Suriname and Aves Island, Venezuela and is the second most common haplotype at Tortuguero, Costa Rica and was found in a single individual in Quintana Roo, Mexico (Figure 4.1; Encalada et al. 1996; Lahanas et al. 1998; Bjorndal et al. 2005). Discerning whether the extensive haplotype sharing among rookeries is attributable to shared evolutionary history with recent isolation or contemporary exchange of females among rookeries is important for assessing population structure for management on ecological time scales. Moreover, the overlap of genetic markers among rookeries has the potential to introduce considerable uncertainty into estimates of rookery contributions to mixed foraging aggregations, even if it is clear that the rookeries are demographically partitioned based on haplotype frequency differences (Bolker et al. 2007).

Defining population structure and migratory connectivity of developmental habitats for Greater Caribbean green turtles is of special significance given the diminished nature of populations in the region. Europeans heavily exploited green turtles in the Caribbean during exploration and settlement of the New World (Parsons 1962). Despite encouraging increasing trends in nest counts for several global green turtle rookeries in recent decades, including two in the Greater Caribbean region (Tortuguero, Costa Rica and Archie Carr National Wildlife Refuge, Melbourne Beach, Florida, USA; (Chaloupka et al. 2008), estimates based on historic nesting population descriptions and harvest records suggest that the contemporary abun-

dance of green turtles in the Caribbean is less than 1% of pre-Columbian numbers (McClenachan et al. 2006). The Cayman Islands may have once supported the largest green turtle rookery in the Greater Caribbean region (King 1982), but two hundred years of intense harvest pressure extinguished the nesting population by the early 19th century (Parsons 1962). Bermuda also once supported a major nesting population. By the end of the 18th century, Bermuda turtle boats had moved on to the Bahamas and Ascension (Wilkinson 1950), suggesting that the local population had collapsed. Low levels of green turtle nesting have been recorded recently in the Cayman Islands ($\leq$ 51 nests annually; Aiken et al. 2001; Bell et al. 2007), at least a portion of which may be attributable to graduates of the Cayman Turtle Farm head-starting program (Bell et al. 2005). However, marine turtle nesting is considered very infrequent in Bermuda currently (Bermuda data in Dow et al. 2007), demonstrating that reestablishment of extirpated or depleted stocks via natural recruitment from other rookeries is not guaranteed over short ecological time scales (Bowen et al. 1992).

Assessing demographic connectivity among nesting populations is of particular concern for the Aves Island green turtle rookery given the history of erosion on the island and harvest of nesting females. Despite roughly 1300 km of separation, the rookeries of Matapica, Suriname and Aves Island were not significantly different with respect to their 490 bp control region haplotype frequencies, although the authors cautioned that this was likely due to recent isolation rather than contemporary exchange of females between rookeries (Encalada et al. 1996). Aves is a remote island approximately 200 km west of Dominica in the Caribbean Sea that serves as important nesting habitat both for seabirds and green turtles (Zuloaga 1955). Ships from several nations once visited the island during nesting season to harvest females as they came ashore to nest. Parsons (1962) estimated that approximately 400 nesting females may have been taken annually during the 1950s. Pinchon reported observing 150 to 200 females nesting nightly during a week-long visit in 1947 (Pritchard and Trebbau 1984), but during the 1972 and 1973 nesting seasons an average of only 22 females emerged per night (Brownell and Guzman 1974). Following recognition of the island as a wildlife refuge

by the Venezuelan government in 1972 (Miloslavich et al. 2003, the population appears to have stabilized recently, with annual nesting female counts in the hundreds (Vera and Guada 2006; Vera 2008). Accounts by visitors to the island suggest a dramatic reduction in surface area and elevation between the sixteenth and nineteenth centuries (Zuloaga 1955). A 1983 survey established that the island was approximately 640 m in length, 30 to 270 m in width, and had a maximum elevation of 3.72 m (Schubert and Laredo 1984). The presence of coarse gravel on the highest points of the island prompted speculation that it may be periodically completely submerged, probably during hurricanes (Schubert and Laredo 1984). Given the vulnerability of the island to erosion, the fate of the Aves nesting population has been the subject of concern and interest for decades; Parsons (1962) noted, "Should it (Aves Island) completely founder, the Aves turtles will be faced with a dilemma of considerable proportions and one that would be of much interest to students of animal behavior." The recent increase in green turtle nesting recorded on Buck Island, USVI (Buck Island Reef National Monument, unpublished data) may be attributable to females straying from Aves Island or recovery of a depleted stock. Investigation of the relationship of the Aves Island rookery with others in the Lesser Antilles is required to characterize the scale of genetic and demographic connectivity in the region.

Nuclear markers offer a fast-evolving alternative to the mitochondrial genome but have yielded mixed results in detecting population structure within and among marine turtle rookeries. Analysis of mtDNA haplotypes frequencies failed to detect any partitioning at the Tortuguero rookery (Bjorndal et al. 2005); however Peare and Parker (1996) reported evidence of genetic structure among green turtles nesting there at a scale of eight km using nuclear markers. The same study failed to detect any structure among green turtles nesting along Melbourne Beach, Florida using nuclear markers (Peare and Parker 1996). Using assignment tests based on five microsatellite loci, Lee et al. (2007) reported a significant signal for precise female natal homing at two of the three sampled green turtle nesting beaches tested at Ascension Island despite a lack of differentiation among beaches as measured by

traditional F statistics based on microsatellites or mtDNA (Formia et al. 2007). Despite the potential of increased resolution with nuclear markers, a limitation of their use to define population structure among marine turtle rookeries is that nuclear surveys have generally detected equivalent or considerably less structure than that inferred using mitochondrial markers at regional spatial scales (hundreds of km) (Karl et al. 1992; FitzSimmons et al. 1997b; Roberts et al. 2004b; Bowen et al. 2005; but see Carreras et al. 2007). Genetic surveys utilizing nuclear markers (RFLP analysis of anonymous single-copy loci and four microsatellites) failed to detect differentiation among Greater Caribbean green turtle rookeries (Karl et al. 1992; Roberts et al. 2004; Wallace et al. 2010) despite marked mtDNA haplotype frequency differences among several rookeries in the region (Encalada et al. 1996). This disparity in signal has been attributed, at least in part, to male-mediated or migration-mediated gene flow in the presence of strong natal philopatry by females (Karl et al. 1992; FitzSimmons et al. 1997b). Marine turtle rookeries flourish or perish based on female recruitment to particular nesting areas, therefore nuclear gene flow should not detract from recognition of rookeries as distinct management units when mtDNA indicates differentiation (Bowen et al. 2005). Therefore, expanded screening of the mitochondrial genome may benefit analyses of genetic structure among rookeries at regional spatial scales where nuclear gene flow is likely to occur via population admixture on foraging grounds or along migratory corridors.

Utilization of additional mitochondrial sequence data has improved resolution of studies of phylogeography and population structure in several migratory marine taxa. Most population structure studies of Atlantic rookeries and foraging aggregations have assigned haplotypes for loggerhead and green turtles based on 380 bp and 490 bp control region sequences, respectively. Haplotype CC-A1 that was shared between western Atlantic and Cape Verde loggerhead turtle rookeries has been subdivided into apparently endemic haplotypes through comparisons of an expanded 760 bp control region fragment (Monzòn-Argüello et al. 2010b). Use of this larger control region fragment has also resulted in increased resolution in studies of Atlantic hawksbill and leatherback turtles (Vargas et al. 2008; Velez-Zuazo et al. 2008;

Browne et al. 2009). Despite sharing control region haplotype CM-A8 with Brazilian and Ascension rookeries (Encalada et al. 1996), green turtles from Guinea Bissau carried a unique restriction digest profile at a DraII site (Bowen et al. 1992), illustrating that additional informative variation occurs outside the established control region fragment. A mitogenomic approach resolved the temporal phylogeography of Atlantic codfish (*Gadus morhua*) and determined that the most widespread haplotype based on cytochrome b sequences was actually a paraphyletic assemblage of diverse mitogenomes (Carr and Marshall 2008). Similarly, forty individuals sampled at four whelping patches of harp seals (*Pagophilus groenlandicus*) across the North Atlantic were dominated by a single common cytochrome b haplotype, and phylogeographic signal was lacking (Perry et al. 2000); however mitogenomic analysis indicated that each individual carried a unique mitochondrial sequence and that deep ancestral clades were present (Carr et al. 2008). Phylogeographic analysis of whole mitochondrial genome sequence variation in killer whales (*Orcinus orca*) provided strong support for species status of the ecotypes (Morin et al. 2010), whereas an earlier analysis based on shorter segments of sequence failed to resolve these relationships because of the limited polymorphism detected (Hoelzel et al. 2002).

To date, marine turtle haplotypes have been assigned based on less than 1 kilobase (kb) of the $>$ 16 kb mitogenome. Undescribed polymorphisms outside the established control region fragments may remedy several intractable cases of haplotype overlap among marine turtle rookeries and improve the resolution of downstream mixed stock analyses. We searched for additional informative variation through sequencing of the majority of the mitochondrial genome (16140 of 16497 bp) of nesting turtles with haplotype CM-A5 and representing the rookeries of Buck Island, United States Virgin Islands (USVI); Aves Island, Venezuela; Galibi, Suriname; and Tortuguero, Costa Rica. The objectives were genetic characterization of the Buck Island rookery in relation to others in the region and reanalysis of population structure among the rookeries using novel sequence variation.

Methods

The samples sequenced in the present study were collected at four green turtle rookeries in the southern Greater Caribbean region: Tortuguero, Costa Rica; Buck Island, USVI; Aves Island, Venezuela; and Galibi, Suriname (Figure 4.2). Samples previously analyzed include: all CM-A5, CM-A20, and CM-A21 individuals sampled at Tortuguero in 2001 and 2002 (n = 37; Bjorndal et al. 2005); Aves Island samples from three previous studies (n = 4, 34, 30 respectively, in Bowen et al. 1992; Roberts et al. 2004; Lahanas et al. 1998); Galibi, Suriname individuals sampled in 1999 and 2000 (n = 58; unpublished data in Bolker et al. 2007). Additional samples were collected from Buck Island from females nesting from 2001 through 2009. These samples were collected using 6-mm biopsy punches and stored in a 20% DMSO saturated NaCl buffer (Dutton and Balazs 1995). Each female was tagged with Inconel tags in both front flippers (Balazs 1999) to ensure that individuals were sampled only once.

PCR reactions for control region amplification were carried out in 20 μl volumes using primers LCM15382 (GCTTAACCCTAAAGCATTGG; Abreu-Grobois et al. 2006) and a novel reverse primer CM16437 (TTGGTTGAGGTGTGGTAGAG). The novel primer was designed to amplify approximately 150 bases beyond the fragment amplified by LCM15382 and the reverse primer H950 (Abreu-Grobois et al. 2006), and extends the fragment to just 5' of the repetitive element in the control region. Additional portions of the mitochondrial genome (the complete genome less the repetitive element in the control region and bases 1-279) were amplified in 25 μl volumes using primers designed from the published green turtle mitochondrial genome (Table 4.1; Kumazawa and Nishida 1999). Reactions contained 10mM Tris, pH 8.4; 50 mM KCl, 0.5 μM of each primer, 1.5 mM MgCl2, 0.25 mM dNTPs, 0.5 unit of *Taq* DNA polymerase, and approximately 10-50 ng of genomic DNA. PCR cycling parameters were as follows: 95°C for 5 minutes; 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 to 90 s depending on fragment length; and a final extension of 72°C for 10 minutes. PCR products were purified using ExoSAP-IT®(USB Corporation) according

to manufacturer's instructions. The control region amplicons were sequenced in a single direction with LCM15382 and an internal forward sequencing primer (Cm15821, TCACGAGAAATAAGCAAC) using ABI BigDye v3.1 (PE Applied Biosystems) and an ABI 3730xl DNA Analyzer. Sequencing reactions for additional portions of the mitochondrial genome were conducted in a single direction using forward PCR primers as well as internal sequencing primers designed from the published green turtle mitochondrial genome (Table 4.1). Negative controls were utilized in each batch of PCR amplification and sequencing reactions to detect contamination. The first round of mitochondrial genome screening was performed using two CM-A5 individuals each from the rookeries of Suriname, Aves Island, and Tortuguero. Following mitogenomic haplotype assignments based on the SNPs detected in the first round of screening, the mitochondrial genomes of 13 additional CM-A5 turtles were sequenced in search of additional polymorphism. The remaining haplotypes were screened only at the SNPs identified for CM-A5 and CM-A3.

Sequences were aligned, edited, and compared to previously described haplotypes and the published green turtle mitochondrial genome using the program Sequencher 4.2 (Gene Codes Corporation). Sequences were assigned haplotype designations after nomenclature published on the Archie Carr Center for Sea Turtle Research (ACCSTR) website (http://accstr.ufl.edu/cmmtdna.html). Mitogenomic haplotype names consist of a series of three numerals corresponding to different fragments of the mitochondrial genome. The first number in the series denotes the original haplotype name based on a 490 bp fragment of the mitochondrial control region. The second number in the series denotes variants based on polymorphisms within the 817 bp control region fragment amplified by LCM15382-H950 (but outside the original 490 bp fragment) that subdivide the original haplotype. Finally, the third number represents variations based on polymorphism outside the 817 bp control region fragment. Samples producing novel or ambiguous sequences were subjected to a second round of DNA extraction, PCR amplification, and sequencing for verification. Novel haplotypes were deposited with Genbank and ACCSTR.

Haplotype frequency-based pairwise F_{ST} comparisons, pairwise exact tests of population differentiation, and analysis of molecular variance (AMOVA) were conducted using the software Arlequin version 3.1 (Excoffier et al. 2005). Significance values for AMOVA were obtained from 10,000 permutations. Exact tests of population differentiation were conducted with 100,000 permutations and 10,000 dememorization steps after the method of Raymond and Rousset (Raymond and Rousset 1995). All analyses were conducted using both the short haplotypes based on a 490-bp fragment of the 5' end of the control region (Allard et al. 1994) as well as the expanded mitogenomic haplotypes assigned in the present study. Significance of exact tests of population differentiation and pairwise F_{ST} comparisons was adjusted using sequential Bonferroni correction (Rice 1989).

Results

Based on the 490 bp fragment, two haplotypes were detected at the Buck Island rookery: CM-A5 and CM-A16. Sequence alignments of the LCM15382 and CM16437 amplicon revealed two additional polymorphic sites outside the established 490-bp control region fragment: an indel within the CM-A5 lineage and a variable position between the haplogroups containing CM-A3 and CM-A5 (Table 4.2). The conserved haplotype has been designated CM-A5.1, and the haplotype with the insertion has been designated CM-A5.2. Sequencing of extra control region fragments revealed four variable positions corresponding to three mitogenomic CM-A5.1 haplotype variants (Table 4.2). Control region haplotypes CM-A20.1 and CM-A21.1 from Tortuguero shared the two derived mutations present in the common CM-A5.1 variant present at Tortuguero, CM-A5.1.2 (Figure 4.3). In addition to the polymorphism detected within the CM-A5 lineage, mitochondrial genome sequence alignments from outside the control region identified seven polymorphic sites that were variable between CM-A5.1.1 and a CM-A3.1.1 nesting female from Tortuguero. The second round of mitogenomic screening did not identify any additional SNPs.

Novel polymorphism identified in this study was highly informative with respect to regional population structure. The conserved mitogenomic variant CM-A5.1.1 was detected in all four rookeries surveyed and was the only variant found at Galibi (Figure 4.2; Table 4.3). CM-A5.1.2 occurred at high frequency at Tortuguero (84% of CM-A5 variants) but was not found elsewhere. CM-A5.1.3 was common at both Aves Island and Buck Island but absent at Galibi and Tortuguero. CM-A5.2 was common in the Aves Island rookery but detected in only a single nesting female at Buck Island and was not found in Galibi or Tortuguero females.

The overall structure partitioned among rookeries by AMOVA using only frequencies of the 490 bp haplotypes was high ($F_{ST} = 0.8081$, $p < 0.00001$). All Tortuguero vs. eastern Greater Caribbean pairwise comparisons were significantly different (Table 4.4). However, none of the eastern Caribbean comparisons were significant with respect to pairwise F_{ST} values, and Aves Island and Galibi were not differentiated based on pairwise exact tests of population differentiation when only the short haplotypes were analyzed (Table 4.4). With mitogenomic haplotypes analyzed the overall structure among rookeries declined slightly because of the increased polymorphism detected within rookeries ($F_{ST} = 0.6961$, $p < 0.00001$). However, all pairwise F_{ST} comparisons and exact tests of population differentiation were significant in analyses using mitogenomic haplotypes (Table 4.4).

Discussion

Population Structure

Comparative mitogenomic analysis revealed that partial control region haplotype CM-A5 is an assemblage of at least four distinct lineages that are subdivided among regional rookeries. Mitogenomic haplotype frequencies were significantly different for each of the four sampled rookeries, suggesting that sufficient demographic partitioning exists to warrant separate management unit status for Buck Island, Aves Island, Suriname, and Tortuguero rookeries. The latter has always been considered genetically distinct from eastern Caribbean rookeries based

on haplotype frequency differences (Encalada et al. 1996), although more thorough sampling at Tortuguero revealed increased haplotype sharing with eastern Caribbean rookeries relative to the initial survey (Bjorndal et al. 2005). Mitogenomic analysis indicated that 84% of the CM-A5 females nesting at Tortuguero belong to a lineage that was not recorded elsewhere in the Greater Caribbean region. Genetic evidence of population subdivision between Aves Island and Suriname rookeries had not been previously detected using partial control region haplotypes, although Encalada et al. (1996) cautioned that the lack of differentiation was likely attributable to recent isolation rather than ongoing gene flow. Mitogenomic data corroborate the hypothesis of demographic isolation of these rookeries.

Mitogenomic comparisons of the Buck Island and Aves Island rookeries indicated significant population structure occurred at the finest spatial scale examined in this study, approximately 250 km. This distinction would have gone undetected through analysis of the 490 bp haplotypes. CM-A5.2, comprising approximately 20% of the Aves Island sample, was detected in only a single individual nesting on Buck Island. CM-A3.1, recorded at low frequency at Aves Island, was absent at Buck Island, despite high sampling effort relative to nesting densities at the latter rookery over the past decade. Additionally, haplotype CM-A16 recorded from four females at Buck Island was not detected among Aves Island females. CM-A16 had not previously been described from the eastern Caribbean, and was known only from Quintana Roo, Mexico rookeries (Encalada et al. 1996). The significant haplotype frequency differences detected between these rookeries suggest that if contemporary demographic connectivity exists, it is likely limited and that the Buck Island rookery warrants recognition as a distinct management unit, probably as part of a larger USVI stock. However, genetic characterization of the high density rookeries of the East End beaches of St. Croix, USVI with the SNPs identified in the present study is required to better assess the connectivity of the green turtle rookeries within USVI and their relationship to the Aves Island rookery.

Phylogeography

Encalada et al. (1996) hypothesized that precursors of haplotypes CM-A5 and CM-A6 colonized the beaches of northeastern South America from equatorial Atlantic refugia. These haplotypes branch from CM-A8, the most common haplotype among equatorial rookeries and the central haplotype in the network of the eastern Caribbean and equatorial Atlantic haplogroup (Encalada et al., 1996). Recent surveys of insular rookeries in western Africa and Ascension Island detected haplotype CM-A6 at low frequency, and one CM-A5 individual was reported from the São Tomè rookery (Formia et al., 2006; Formia et al., 2007). These surveys also detected haplotypes CM-A35 and CM-A39, which likely descend from CM-A6, at the São Tomè and Ascension rookeries, respectively. All four haplotypes were notably absent in surveys of the Brazilian rookeries of Atol das Rocas and Trindade Island (Bjorndal et al., 2006). These data suggest a possible central or eastern Atlantic origin of the precursors of the CM-A5 lineage that colonized Suriname and neighboring coasts rather than their origination from proximal Brazilian rookeries. Among the mitogenomic variants of CM-A5 detected in the present study, CM-A5.1.1 was central within the haplotype network and was recorded from all four Greater Caribbean rookeries analyzed. These findings support the hypothesis that within the Greater Caribbean region, the CM-A5 lineage colonized northward and westward from Suriname.

Two derived mitogenomic CM-A5 variants were present in the Aves Island and Buck Island rookeries, but both were absent among other sampled rookeries. Historical green turtle nesting in the USVI was characterized as "minor" relative to the high nesting densities at Aves Island (McClenachan et al. 2006). Therefore, the two major lineages nesting on Buck Island may ultimately descend from the Aves Island population. Given the erosional nature of Aves Island (Schubert and Laredo 1984), straying may have occurred during a period of inundation when no suitable nesting habitat was available. The nearest islands to Aves Island are those of the Lesser Antilles more than 175 km distant. In addition to St. Croix and Buck Island, USVI, St. Eustatius and Guadeloupe host regular green turtle nesting

in low numbers (< 100 crawls per beach per year; Dow et al. 2007). Still lower numbers of green turtle nests are recorded from several other islands of the Lesser Antillean chain. Genetic characterization of these smaller rookeries is needed to better characterize spatial and temporal scales of demographic connectivity of green turtle rookeries in the region.

The presence of CM-A5.1.1 in addition to the derived variants found at Tortuguero, Buck Island, and Aves Island may result from incomplete lineage sorting or multiple colonization events by turtles of the CM-A5 lineage at these sites. That Tortuguero haplotypes CM-A20.1 and CM-A21.1 share the two mutational steps that distinguish CM-A5.1.2 from the conserved variant suggests the latter scenario may be more likely, at least for that rookery. Use of highly polymorphic nuclear markers may elucidate whether the CM-A5.1.1 females nesting at Tortuguero are of recent common origin or may themselves represent multiple straying events. The colonization of Tortuguero by the CM-A5 lineage, likely from distant eastern Caribbean sources, prompts the question as to the mechanisms underlying nesting beach selection when "mistakes" in natal homing occur.

Colonization of a western Caribbean rookery by an eastern Caribbean lineage may have been facilitated in part by presence of CM-A5 turtles of eastern Caribbean origin in the foraging aggregations near the nesting beach at Tortuguero. Mark-recapture data suggest that green turtles recruit through multiple neritic developmental habitats (Moncada et al. 2006; Bjorndal and Bolten 2008; Senko et al. 2010) before ultimately choosing an adult foraging ground to which they show high site fidelity (Limpus et al. 1992). Tortuguero nesting females are known to forage primarily along the coast of Nicaragua (Carr et al. 1978; Bass et al. 1998; Troëng et al. 2005), which also serves as a major foraging ground for females that nest on Aves Island (Sole 1994). The precise mechanisms that trigger the ontogenetic shift from pelagic to neritic foraging, as well as those that facilitate recruitment to novel developmental foraging sites are not fully understood. The distribution of turtles on foraging grounds probably results from complex interactions of ocean currents and turtle behavior (Bass et al. 2006). Tag return data from large juvenile green turtles in Nicaraguan waters

suggest that the sea grass beds also serve as important developmental habitat for subadults (Moncada et al. 2006). Therefore, large juvenile turtles from the Aves Island rookery may be present along the Nicaraguan coast in close proximity to the nesting beach at Tortuguero.

Proximity of foraging turtles to a non-natal nesting beach is insufficient to explain aberrant nesting beach selection in the absence of other mechanisms. One possible scenario to account for nesting of the eastern Caribbean lineage in the western Caribbean is that the beach at Tortuguero may have similar one-dimensional magnetic properties to Aves Island. Marine turtles are thought to imprint on the geomagnetic signature of their natal beach and use this positional information to home back to their natal region for nesting (Lohmann et al. 2008b). Marine turtles can detect changes in magnetic inclination and intensity (Lohmann et al. 2007), and experiments have suggested that loggerhead turtles are capable of perceiving longitude as part of a bicoordinate magnetic mapping system (Putman et al. 2011). Such bicoordinate maps may be particularly important for navigating to the vicinity of islands, at which point other cues likely facilitate the final stage of fine scale nesting beach selection (Lohmann et al. 2008a; Lohmann et al. 2008b). Should errors in the perception of the bicoordinate magnetic map occur, turtles may rely on portions of the magnetic map (eg. magnetic intensity signatures as a surrogate of latitude; Lohmann and Lohmann 1994) while following along a continental coastline that would behave as a fixed longitudinal coordinate (Lohmann et al. 2008b). The magnetic signatures of field intensity are similar for Tortuguero and Aves Island based on maps of 1 μT contour interval isolines (Macmillan and Maus 2005); therefore a neophyte reproductive female of Aves Island origin leaving the foraging grounds in Nicaragua may have arrived at Tortuguero using only a partial magnetic signature that approximates latitude.

Social facilitation may also play a role in nesting beach selection when the normal mechanisms responsible for natal homing break down. Under the social facilitation model, virgin females follow experienced nesters from foraging grounds to the nesting beach and imprint on the site following a positive nesting experience (Hendrickson 1958; Owens et al. 1982).

Though genetic evidence has refuted social facilitation as a primary force driving nesting beach selection in green turtles and other marine turtle species (reviewed in Bowen and Karl 2007), it is conceivable that a "lost" neophyte nesting female originating from the Aves rookery might have found the beach at Tortuguero by following a female of that population from shared foraging grounds.

Mitogenomic sequencing for population structure assessments

Several studies have demonstrated the utility of complete mitogenomic sequencing for resolving problematic nodes and producing more robust estimates of divergence times in a phylogenetic context (eg. Inoue et al. 2001; Zhang et al. 2004; Pereira and Baker 2006). Mitogenomic sequencing has also proven beneficial in improving genetic signal in intraspecific phylogeographic studies (Ingman et al. 2000; Carr and Marshall 2008; Carr et al. 2008; Morin et al. 2010; Stone et al. 2010; Wang et al. 2010). The present study extends the utility of mitogenomic sequencing for population structure analyses in a taxon with low levels of nucleotide diversity within haplogroups and shallow evolutionary population structure within ocean basins (Bowen et al. 1992; Encalada et al. 1996). Control region haplotype sharing among rookeries is a common problem in all marine turtle species with cosmopolitan distributions. Phylogeographic and population structure assessments as well as mixed stock analyses of several marine turtle taxa could benefit from mitogenomic SNP discovery and analyses. Clearly, some overlap of haplotypes among rookeries remains despite the expanded sequencing effort. This haplotype sharing may never be fully resolved given marine turtle dispersal capability and inferred slow rate of mtDNA evolution (Avise et al. 1992). Nonetheless, the present study demonstrates the utility of mitogenomic SNPs for detecting cryptic structure among populations that are marked by extensive sharing of a common haplotype based on < 1 kb of the mitogenome.

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Table 4.1: Characterization of primers for mitogenomic fragments analyzed for southern Greater Caribbean green turtles.

	PCR Primers	Sequencing Primers
Fragment 1	Cm00016F: TATATTAAAGCACGGCACTG Cm01472R: GCGTTAGGCTTTTCACC	Cm00477: AATTTGAACTCACGATTG Cm00847: TGAAATAGGTCCCACAAG
Fragment 2	Cm01280F: GCCACAAAAAGCAAAGATT Cm02284R: AACCAAAAATATAGGTCAAGTAC	Cm01657: GCCACCGTCAAAGAGA Cm01949: TAACTGAACCCGACTGT
Fragment 3	Cm02054F: GTGCAAGGGTAGCGTAATC Cm03468R: GCCAGCAGCGTATTCAAC	Cm02444: CGACGATGGGGTTTAC Cm02857: ATCGCCGTCGCTTTC
Fragment 4	Cm02999F: ACCCATCAAACTCATCCAT Cm04574R: TTTGGGGGATAAAGTAAGG	Cm03414: TACCGAAGGAGAATCTGA Cm03821: TAAAATCCCCTCGTCTC Cm04125: CCACGAGCAACTGAAG
Fragment 5	Cm04298F: ACTCGCCCCATTCCACT Cm05491R: TGCCGACTATTCCTGCTC	Cm04563: TTATCCCCCAAACTTATA Cm04926: CACAAAAACAACCAGAAAC
Fragment 6	Cm05247F: TCTATAAAAAGCGGGAAAAC Cm06596R: AGAGGGAATCAGTGGGTAAA	Cm05632: TTCGGAAATTGACTTGTT Cm06053: CCTTCTTCGACCCTTC
Fragment 7	Cm06427F: GGCTTCATTTTCCTCTTTAC Cm07534R: CGGATGGGTGATTCTATTG	Cm06860: TCACAACCACCAACGTA
Fragment 8	Cm07240F: CCAATACTATAAATGCTCAAGAA Cm08762R: AGTAGTGCTGCTGCTGCTC	Cm07637: TGTTACACGACCAGGAGT Cm07983: ACTCCTGAACCTGACCA Cm08386: CTACCAGAAGGCACTCC
Fragment 9	Cm08620F: GCCATAATCCAAGCCTAC Cm09996R: TAATGATAAAGGCGGAGTG	Cm09017: AGGAGGATGTTGACCAC Cm09364: CCTCCACTCACCACTTC
Fragment 10	Cm09778F: CTCCCATCCCCAACTCT Cm11097R: GATTTTAGGTCTGTTTGTCGT	Cm10010: CTCACTACACCGAACTCAT Cm10383: AACCAACCATAAACTTCTC Cm10651: ATGAGGTAATCAAATAGAAC
Fragment 11	Cm10889F: TGATTACCAAAAGCACACG Cm12242R: GAATATGTTGTTGGCTGTTACTA	Cm11385: CCATTATTGCCTCACTGT Cm11747: CCTCAGCTCCCTCACT
Fragment 12	Cm11931F: AAAACAAAAACAGCCGTAA Cm13495R: CAGGATAGGTCGATTAGGTG	Cm12351: AACCGCATAGGAGACAT Cm12781: GCCTAAATCAACCACAA Cm13081: TTACCGCAATCTACAGTC
Fragment 13	Cm13360F: CAACCCAAAAACCCTCTAA Cm14715R: GTACTAGTGTGTTGCCGATGT	Cm13784: AACAACCCACCAGCAT Cm14160: TTCTTGCCTGGACTTTA
Fragment 14	Cm14596F: TAGTCATAGCTACCGCATTC Cm15532R: CACTTTTATTCCTGTGTCAAA	Cm14949: CTAACCTTAACACTTTTCTC

Table 4.2: Variable positions for southern Greater Caribbean green turtles on CM-A5 and CM-A3 mitochondrial genome sequencing. Numbers correspond to base locations in the established control region alignments and the published mitochondrial genome. Equatorial and Greater Caribbean haplogroup variable positions are based on comparisons of sequenced CM-A5 individuals with two CM-A3.1 nesting females from Tortuguero. The light gray shading indicates variable positions outside the control region. The dark gray shading indicates novel control region variation found using LCM15382 and CM16437. NA indicates positions that were not analyzed.

	490 bp												81	84	121
	817 bp												160	163	200
Haplotype	genome	4107	4301	5463	8528	9263	10745	11554	13388	14288	14726	14729	15608	15611	15648
CM-A5.1.1		A	T	C	C	T	C	T	T	C	A	G	C	G	A
CM-A5.2		.	.	.	.	.	.	.	.	.	.	.	.	.	.
CM-A5.1.2		.	.	.	.	.	T	.	C	.	.	.	.	.	.
CM-A5.1.3		.	.	.	.	.	.	.	.	.	G	.	.	.	.
CM-A6.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.
CM-A20.1		.	.	.	.	.	T	.	C	.	.	.	.	.	.
CM-A21.1		.	.	.	.	.	T	.	C	.	.	.	.	.	.
CM-A16.1		.	.	T	.	.	.	C	.	.	.	.	T	A	G
CM-A3.1.1		G	C	T	T	C	.	C	.	T	.	A	T	A	G
CM-A4		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	T	A	G

	490 bp	137	161	238	260	272	353	355	398	419	421	485		
	817 bp	216	240	317	339	351	432	434	477	498	500	564	692	811
Haplotype	genome	15664	15688	15765	15787	15799	15880	15882	15925	15946	15948	16002	16130	16248
CM-A5.1.1		G	A	C	T	G	C	A	C	G	A	G	T	-
CM-A5.2.1		.	.	.	.	.	.	.	.	.	.	.	.	C
CM-A5.1.2		.	.	.	.	.	.	.	.	.	.	.	.	-
CM-A5.1.3		.	.	.	.	.	.	.	.	.	.	.	.	-
CM-A6.1		.	.	.	.	.	.	.	.	.	.	A	.	-
CM-A20.1		.	G	.	.	.	.	.	.	.	.	.	.	-
CM-A21.1		.	.	.	.	.	.	.	T	.	.	.	.	-
CM-A16.1		A	.	.	C	T	T	G	T	A	G	A	C	-
CM-A3.1.1		.	.	T	.	T	.	G	T	A	G	A	C	-
CM-A4		.	.	T	.	T	T	G	T	A	G	A	NA	NA

Table 4.3: Mitogenomic haplotype frequencies for southern Greater Caribbean green turtle rookeries. CM-A3 and CM-A4 counts are from published data based on partial control region sequences only (Encalada et al. 1996; Bjorndal et al. 2005).

	Tortuguero	Buck Island	Aves Island	Galibi
CM-A3	395		6	1
CM-A4	1			
CM-A5.1.1	5	23	27	55
CM-A5.1.2	27			
CM-A5.1.3		22	21	
CM-A5.2		1	14	
CM-A6.1				2
CM-A16.1		4		
CM-A20.1	2			
CM-A21.1	3			

Table 4.4: Pairwise F_{ST} values (above the diagonal) and p values from exact tests of population differentiation (below the diagonal) among southern Greater Caribbean green turtle rookeries. Values with parentheses were generated from analysis of 490-bp control region haplotypes. Asterisks indicate significant pairwise F_{ST} comparisons at $\alpha = 0.05$ with sequential Bonferonni correction for multiple tests.

	Tortuguero	Buck Island	Aves	Suriname
Tortuguero		0.747* (0.828*)	0.671* (0.809*)	0.843* (0.834*)
Buck Island	< 0.00001 (<0.00001)		0.034* (0.027)	0.392* (0.016)
Aves	< 0.00001 (<0.00001)	0.0002 (0.0044)		0.336* (0.012)
Suriname	< 0.00001 (<0.00001)	<0.00001 (0.0203)	< 0.00001 (0.0651)	

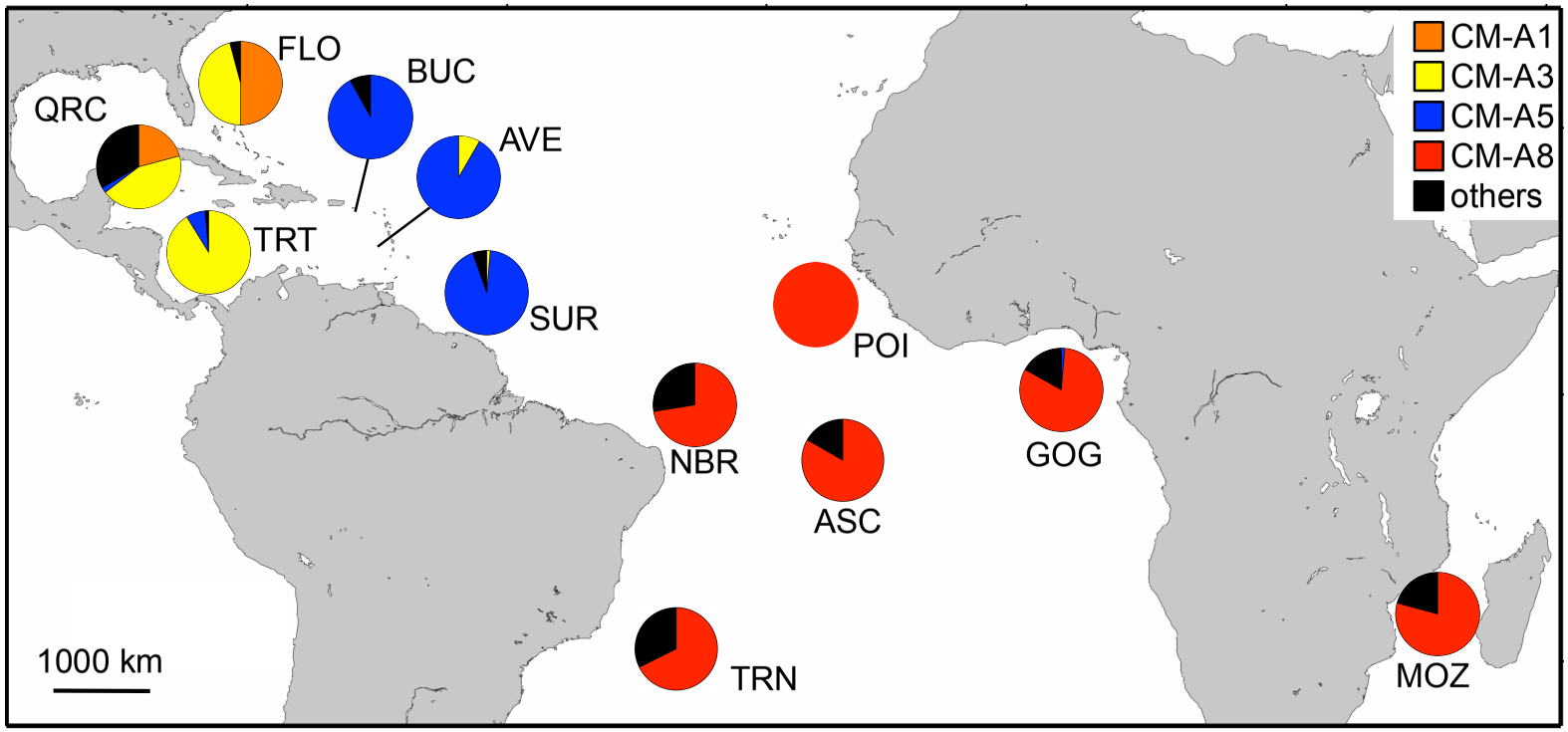

Figure 4.1: Locations and frequency distribution of common 490-bp mitochondrial control region haplotypes for select Atlantic and southwest Indian Ocean green turtle rookeries. Some distinct management units were pooled for legibility. FLO, Florida, United States of America (Encalada et al. 1996); QRC, combined Quintana Roo, Mexico and southwestern Cuba (Encalada et al. 1996, Ruiz-Urquiola et al. 2010); TRT, Tortuguero, Costa Rica (Encalada et al. 1996; Bjorndal et al. 2005); BUC, Buck Island, United States Virgin Islands (present study); AVE, Aves Island, Venezuela (Encalada et al. 1996, Lahanas et al. 1998; present study); SUR, combined Matapica and Galibi, Suriname (Encalada et al. 1996; present study); POI, Poilão, Guinea Bissau (Formia et al. 2006); NBR, combined Atol das Rocas and Fernando de Noronha (Encalada et al. 1996; Bjorndal et al. 2006); ASC, Ascension Island (Encalada et al. 1996; Formia et al. 2006; Formia et al. 2007) GOG, combined Bioko and Corisco, Equatorial Guinea as well as São Tome and Principe (Formia et al. 2006); TRN, Trindade Island, Brazil (Bjorndal et al. 2006); and MOZ, combined Juan de Nova and Europa (Bourjea et al. 2007).

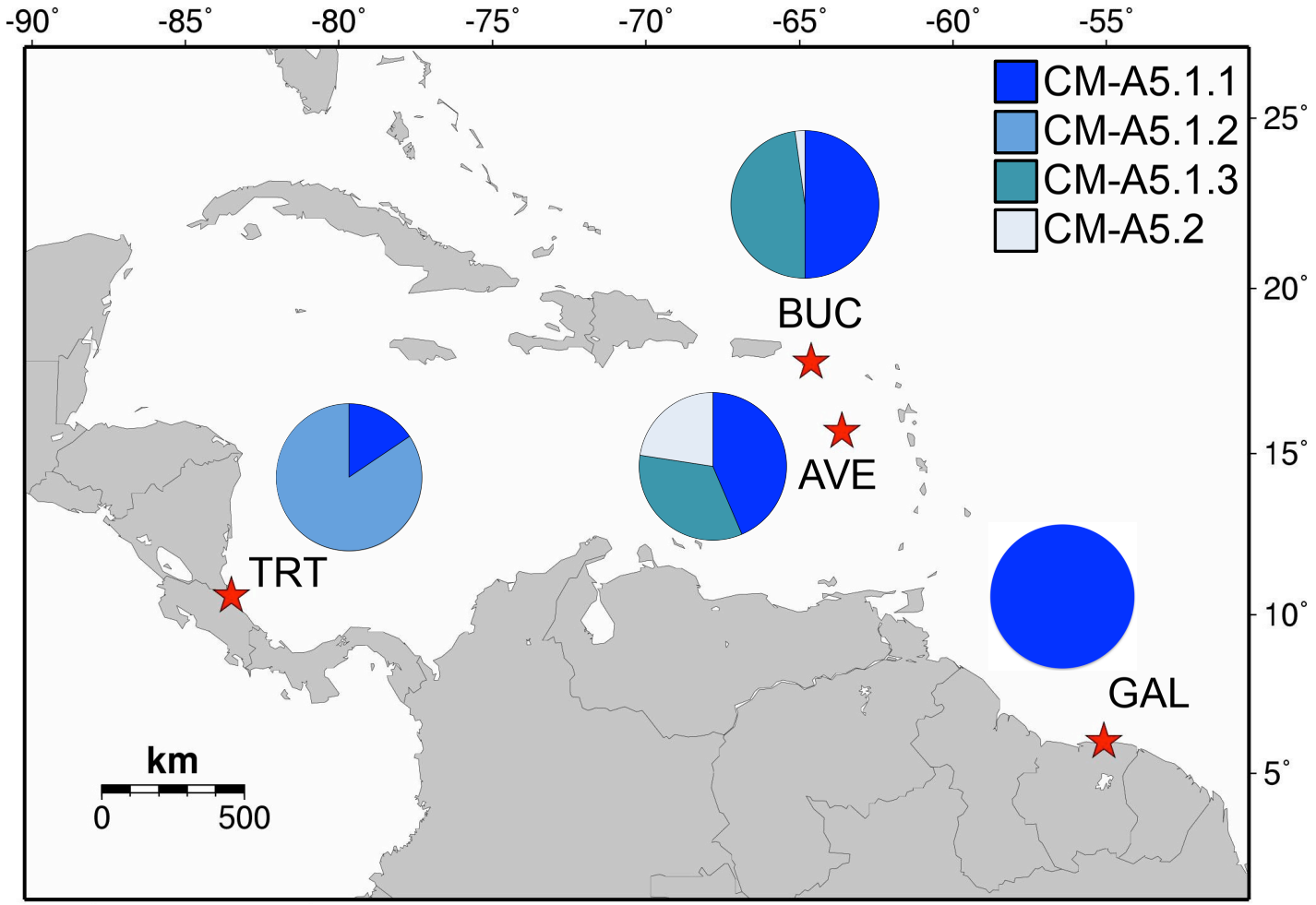

Figure 4.2: Locations and frequencies of mitogenomic CM-A5 haplotypes for select green turtle rookeries in the southern Greater Caribbean region: TRT, Tortuguero, Costa Rica; BUC, Buck Island, United States Virgin Islands; AVE, Aves Island, Venezuela; and GAL, Galibi, Suriname.

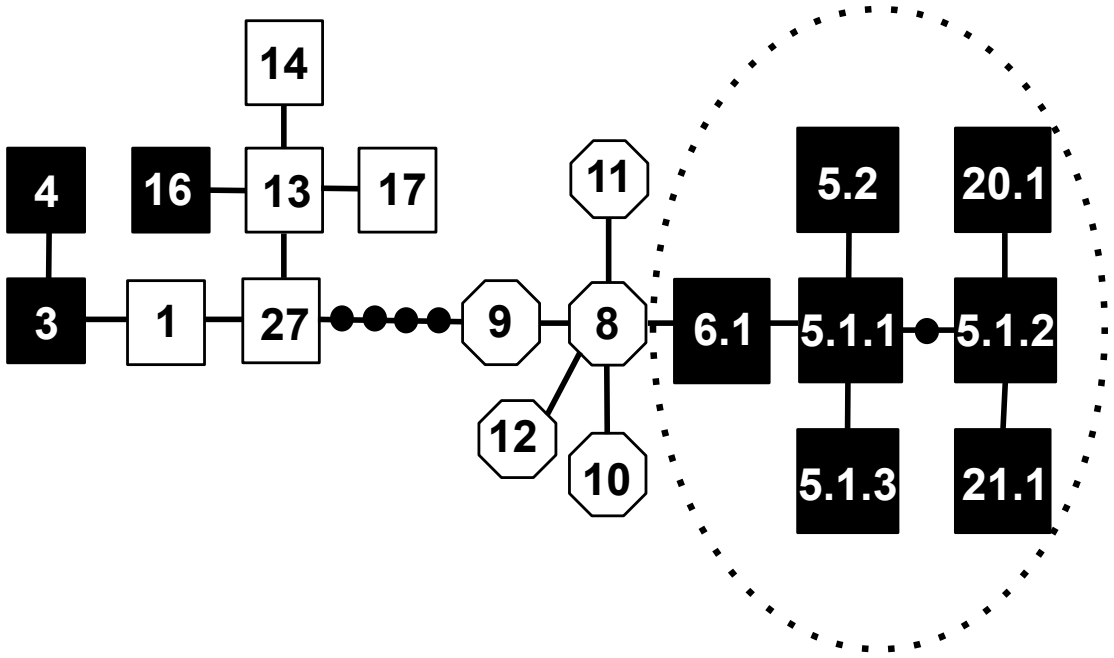

Figure 4.3: Haplotype network modified from Bjorndal et al. (2005) illustrating common Atlantic green turtle control region haplotypes based on the 490 bp fragment. Haplotypes encircled by the dotted line illustrate mitogenomic variation within the CM-A5 lineage. Greater Caribbean and Mediterranean haplotypes are in squares; equatorial Atlantic haplotypes are in hexagons. Filled circles represent hypothetical haplotypes. Haplotypes in black were identified at the four rookeries in the present study. "CM-A" prefixes suffixes are excluded for legibility.

Chapter 5

Conclusions

Haplotype frequency differentiation supports the presence of at least seven loggerhead turtle management units along the coast of the southeastern United States of America (USA). Analyses based on larger sample sizes collected over multiple nesting seasons also indicate demographic partitioning of the Gulf and Atlantic coasts of Florida. In addition to the phylogeographic break inferred in the vicinity of Cape Canaveral (Avise 1992; Encalada et al. 1998), a strong genetic break also apparently occurs somewhere between Melbourne Beach and Juno Beach for this species. Finer scale analysis of the intervening rookeries is required to refine management unit boundaries and better resolve the scale of natal philopatry in the nesting aggregation.

Ideally, genetic data should be interpreted in the context of demographic data. Where available, mark-recapture records are generally concordant with results from genetic analyses. Tagging and genetic data suggest that like nest site fidelity, the geographic scale of natal philopatry may vary among species and possibly even among populations reviewed in (Bowen and Karl 2007). Tagging studies detected female nesting dispersal among individual beaches hosting northern and southern Great Barrier Reef green turtle rookeries but not between the two regions. These regions share some haplotypes, but host genetically distinct management units based on strong haplotype frequency differentiation (Dethmers et al. 2006). Low levels of female nesting dispersal have been recorded for loggerhead turtle rookeries along the coast of Quintana Roo, Mexico, consistent with haplotype frequencies suggesting no differentiation among rookeries on mainland beaches or Cozumel (Nielsen 2010). Nesting dispersal appears to be more common among leatherback turtles nesting at mainland leatherback

turtle rookeries compared with those of insular nesting populations in the Caribbean region, also consistent with genetic data that indicate lack of differentiation among mainland sites separated by several hundred kilometers (Dutton et al. 1999).

Melbourne Beach hosts the only active long-term loggerhead turtle tagging project on the east coast of Florida. However, previous tagging projects were conducted at Canaveral National Seashore-Kennedy Space Center, the Sebastian Inlet area, Hutchinson Island, and Jupiter Island in addition to Melbourne Beach (Worth and Smith 1976; Ehrhart 1980; Bjorndal et al. 1983). These projects varied with respect to beach coverage, time frames, and sampling intensity, hindering the estimation of absolute levels of nesting dispersal. However, they do offer a qualitative assessment of the geographical scale and degree of nest site fidelity. Most nesting beach relocation occurred between immediately adjacent beaches in Volusia and Brevard Counties (Ehrhart 1980). However, several individuals were also recorded moving between Melbourne Beach and Jupiter Island (Bjorndal et al. 1983), which is approximately 10 km north of Juno Beach and likely to have a similar haplotype profile.

The mark-recapture data are consistent with historical levels of "straying" (nesting at non-natal sites) sufficient to permit colonization of all available suitable nesting habitat along the Atlantic coast of Florida. Theory suggests that selection for philopatry should increase with decreasing availability of suitable breeding habitats (Travis and Dytham 1999). Therefore selection pressure for strong natal philopatry likely occurs for some rookeries, such as the Ascension Island green turtle nesting colony, located thousands of kilometers from the nearest suitable nesting habitat (Carr and Hirth 1962). Conversely, the coast of the southeastern USA represents several hundred kilometers of essentially continuous suitable nesting habitat. Imprecise natal homing would not necessarily result in lower fitness for the straying females. Nonetheless, it is also clear that although levels of straying have been sufficient to permit colonization of all sandy beaches with appropriate thermal incubation qualities throughout much of the southeastern USA, straying has not been so pervasive as to homogenize haplotype frequencies among regions.

Use of the expanded control region haplotypes did not significantly improve structure ata regional level but did improve genetic signal at greater spatial scales. The full utility of these haplotypes will not be realized until they are employed in mixed stock analyses. The dramatic increase in differentiation in western Atlantic and Cape Verde pairwise comparisons using the 817 bp versus 390 bp sequences (eg. northern management unit and Cape Verde, $F_{ST} = 0.273$ with 390 bp and $F_{ST} = 0.736$ with 817 bp) should result in better resolution of rookery contributions where individuals from both nesting aggregations may be mixed, such as the Mediterranean. Although the expanded control region sequences failed to significantly improve the resolution of regional assessments, their utility for resolving population structure in a larger context justifies their continued use, even for small-scale regional analyses.

Extensive haplotype sharing among demographically isolated loggerhead rookeries persists despite use of the expanded control region fragment. One approach to surmounting limitations of the currently established control region fragments is to sequence the entire mitochondrial genome in search of informative variation. Mitogenomic single nucleotide polymorphism discovery resulted in improved discrimination of stock structure among southern Greater Caribbean green turtle rookeries (Chapter 4) that should also translate to increased resolution of mixed stock analysis utilizing the novel genetic variation. It is possible additional polymorphism hidden in the mitogenome may resolve finer scale population structure among southeastern USA rookeries. It is more likely that novel polymorphism could reinforce the distinction between rookeries already recognized as distinct management units. However the green turtle pilot study also demonstrated that mitogenomic sequencing is not likely to fully resolve instances of rookery haplotype sharing in all cases, even at spatial scales at which ongoing demographic connectivity is not suspected to occur.

An alternative means of quantifying rookery connectivity is through individual-based projects. These have been limited to satellite telemetry and flipper-tagging studies until recently. Refinement of genetic techniques has enabled a new avenue of research. Egg shells taken from nests at oviposition were found to contain sufficient quantities of maternal

genomic DNA to amplify microsatellite loci (Shamblin et al. 2011). In combination with a suite of highly polymorphic microsatellite markers isolated from loggerhead turtles (Shamblin et al. 2007; Shamblin et al. 2009), this technique has permitted individual identification and thus genetic mark-recapture of unobserved females nesting along the coast of Georgia. Genetic mark-recapture methods offer the possibility of tracking nest site fidelity of individual turtles through time on large spatial scales more congruent with the scale of nest site fidelity in this species. This technique offers the added advantage of permitting tests of relatedness that could ultimately be used to assess recruitment in long-term studies.

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