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Determination of the Natal Origin and Genetic Stock Composition of a Juvenile Feeding Population of the Loggerhead Turtle (*Caretta caretta*) in Chesapeake Bay

Jeffrey Worner Norrgard
College of William & Mary - Arts & Sciences

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DETERMINATION OF THE NATAL ORIGIN AND GENETIC STOCK
COMPOSITION OF A JUVENILE FEEDING POPULATION OF THE
LOGGERHEAD TURTLE (*CARETTA CARETTA*) IN CHESAPEAKE BAY

A Thesis

Presented to

The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of
Master of Arts

by

Jefferey W. Norrgard

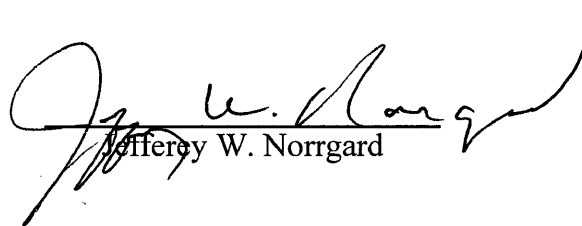
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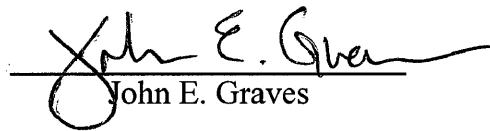
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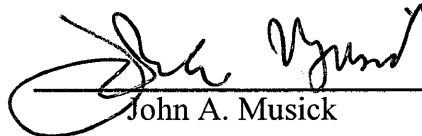
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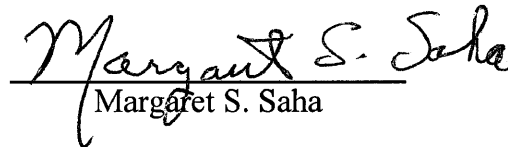
Master of Arts


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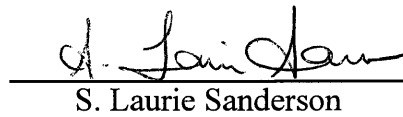

S. Laurie Sanderson

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Abstract

Mitochondrial DNA analysis was employed to assess the relative contribution of two United States rookeries to the aggregate of juvenile loggerhead turtles (*Caretta caretta*) that feed in Chesapeake Bay during the summers. Restriction fragment patterns of the mitochondrial D-loop amplified by the polymerase chain reaction were obtained for 62 individuals collected from 1989-1994. Two haplotypes were found, both characteristic of rookeries in Georgia/South Carolina and Florida. Analysis revealed the Chesapeake Bay population to be a composite of turtles, 69% of which were designated haplotype B and 31% of which were designated haplotype D. Of the individuals comprising this study, 46% were recruited from Florida and 54% from Georgia/South Carolina. Because only 10% of western Atlantic loggerhead nesting occurs in Georgia/South Carolina, these data indicate that turtles from this rookery are selecting the waters of the Chesapeake Bay as juvenile foraging grounds more frequently than their southern counterparts. With the entire western Atlantic loggerhead population in long-term decline, and the northern nesting population more threatened than the Florida nesting population, the Chesapeake Bay population should be protected by wildlife management agencies at a level similar to that of other severely threatened species.

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Introduction

Life history of the loggerhead turtle

Five of the seven recognized species of marine turtles occur within Virginia's estuarine and marine waters (see Table 1). While listed as "threatened" on the U.S. List of Endangered and Threatened Wildlife and Plants, the loggerhead (*Caretta caretta*) is the most frequently encountered sea turtle in the Chesapeake Bay. Each year between 2,000-10,000 individuals migrate into the lower Bay between April and May (Keinath et al., 1987), remaining in the region until September to November, when cool water temperatures force them to migrate south (Lutcavage and Musick, 1985). While wintering grounds are unknown, loggerheads exiting Chesapeake Bay have been tracked as far south as the Florida Keys (Keinath, 1994). The Chesapeake Bay population of turtles consists mainly of juveniles, most with carapace lengths of 60-90 cm, and weighing 25-140 kg (Musick, 1988). Within the Bay their diet consists almost exclusively of horseshoe crabs (Lutcavage and Musick, 1985).

Migration is an important and complex component of the loggerhead's life history, and has been studied extensively using tag and recapture methods, analyses of carapace epibiota, and heavy metal concentration within tissues (Caine, 1986; Stoneburner et al., 1980; Eckert and Eckert, 1988). Gravid females leave their coastal adult foraging

grounds to lay a clutch of more than 100 eggs on a specific nesting beach (rookery). Eggs need more than sixty days at temperatures greater than 25° C in order to incubate successfully (Buckley et al., 1982; McCoy, 1980). Since cooler temperatures tend to increase the ratio of males to females (Standora and Spotila, 1985), the northward spread of successful rookeries is likely a product of recent colonization events over the last 12,000 years. If these rookeries are recent range expansions, then it would be expected that they would exhibit less within-rookery genetic diversity than more established rookeries to the south. This hypothesis has been supported by the genetic studies of Bowen (1993a).

Hatchlings emerge after about sixty days of incubation, and head for major oceanic currents such as the North Atlantic gyre (Figure 1), where they circulate two years or more before recruiting to coastal neritic zones such as the Chesapeake Bay (Carr, 1986). Juvenile loggerheads remain in separate foraging grounds from adults until the onset of sexual maturity, at 20-30 years of age (Klinger and Musick, 1994; Limpus, 1979; Zug et al., 1986). They then migrate to permanent adult feeding grounds, from which females migrate distances up to thousands of kilometers to nest every few years (Margaritoulis, 1988), for a reproductive lifespan of up to 30 years (Frazer, 1983). Male loggerheads spend their entire lives at sea; mating takes place at feeding grounds, along migration corridors, and just offshore of nesting beaches (Bowen and Avise, 1995).

Tagging experiments indicate that adult female loggerheads return repeatedly to the same site to nest (Bjorndal et al., 1983). Three explanations for this nest-site fidelity have been offered over the years. One possibility is that the nesting site is the natal origin

of the female; in other words, natal homing is occurring (Carr, 1967). Another possibility is that females ready to breed for the first time follow experienced females from their adult foraging grounds to rookery sites, and after successfully laying a clutch, return to the same site to lay future clutches (Hendrickson, 1958; Owens et al., 1982). A third option to explain the return rate of females to the same nesting site is that the first time breeder randomly encounters suitable nesting habitat, and after successful clutch laying, fixes on it for future nesting as well (Bowen and Avise, 1995).

Genetic studies

Genetic studies are capable of testing the natal homing hypothesis in sea turtles by yielding a view of population structure that morphological studies are sometimes incapable of providing. Molecular genetic techniques to analyze population structure have developed and progressed rapidly since they were first used nearly 35 years ago. Electrophoresis of water soluble proteins (allozyme analysis) surveys charge and major shape differences, and has been used extensively to study variation within and across species for 30 years. This method is capable of revealing high variation within some species, but not in others. To show population structure in cases where allozyme analysis does not reveal sufficient variation, a more variable character must be studied. In these cases, analysis of nuclear DNA or mitochondrial DNA (mtDNA) can be used. In the early 1980's, restriction fragment length polymorphism (RFLP) analysis was developed to focus on intraspecific differences. RFLP analysis locates the gain and loss of restriction sites within a DNA molecule as subpopulations of the same species diverge over time.

Within species, the development of composite genotypes, or haplotypes, for each individual studied in a population is central to determining population structure.

Individual restriction fragment patterns resulting from a specific restriction endonuclease are referred to as restriction morphs. Haplotypes are produced by grouping restriction morphs for each individual. In the past decade, direct sequencing of genomes has become possible, and is used in many population genetics studies. While nuclear DNA studies allow a view of the total population in question, the use of mitochondrial DNA focuses on female-mediated gene flow within a population. Both offer researchers a genome containing rapidly and slowly evolving regions, which can be chosen for study based on the resolution of population structuring desired.

Mitochondrial DNA is a small circular, double-stranded, polynucleotide sequence located inside the mitochondria. Loggerhead mtDNA is 16.6 kilobases (kb) long (Bowen et al., 1993a). In vertebrates, mtDNA codes for two ribosomal RNA molecules, 22 transfer RNA molecules, and 13 polypeptides, each of which is involved in electron transport or synthesis of ATP (Wilson et al., 1985). It is made up entirely of coding regions (no introns), and is not wound around histones. The displacement loop (D-loop) is the site of the origin of replication in mtDNA. Within this control region are blocks necessary for replication which are highly conserved across species, interrupted by extremely variable regions in terms of sequence content and size (Anderson et al., 1981).

Mitochondrial DNA is inherited almost exclusively maternally; any paternal inheritance in vertebrates is negligible (Wilson et al., 1985). Because of its strict maternal inheritance in sea turtles, mtDNA is ideal for studying the question of natal

homing. If natal homing occurs, there should be low levels (or none at all) of female-mediated gene flow between nesting colonies. Recent mtDNA analysis (Bowen et al., 1993a) of loggerheads in the Atlantic shows significant differences in haplotype frequencies, indicating restrictive gene flow between regional populations, offering solid support for the contention that the nesting location is also the natal origin. This natal homing is not just a loggerhead phenomenon; similar studies confirm that it occurs in both green and hawksbill turtles as well (Allard et al., 1994; Broderick, 1994). All turtles from a single nest have mtDNA genomes identical to their mothers, and over relatively short evolutionary time periods, all turtles at a nesting beach have similar mtDNA (since new rookeries are a product of natal homing "mistakes" by a single nesting female). This similarity allows a researcher to identify the natal origin of any group of individuals by comparing their mtDNA haplotype frequencies to the frequencies of known rookeries. In effect, mtDNA is a natural tag that provides a different perspective than traditional tagging and tracking methods, which have to overcome long generation lengths, pelagic habitat, and long distance movements.

It is well documented that mtDNA, while conservative with regard to gene order and composition, evolves up to ten times faster than single copy nuclear DNA (Brown et al., 1979; Wilson et al., 1985), possibly because mtDNA replicates at a higher rate than nuclear DNA, providing more chances for errors in transcription (Brown et al., 1979). Through restriction site and nucleotide sequence analyses, the rate of mtDNA evolution in sea turtles has been determined to be several times slower than the typical vertebrate estimate (mean rate of divergence of 0.25% and 2% per million years, respectively),

though still significantly faster than nuclear DNA (Awise et al., 1992; Bowen et al., 1993b). It is due primarily to these qualities of maternal inheritance and relatively rapid evolutionary rate that mtDNA has been such a powerful and capable tool in resolving population structure and phylogenetic differences in a variety of studies (Awise et al., 1979; Awise et al., 1984; Hallerman and Beckman, 1988; Yokenow et al., 1981).

In genetic studies on threatened species, it is necessary to avoid harming the populations being sampled by taking small tissue or blood samples (which contain relatively low quantities of genetic material) rather than sacrificing whole individuals. When only very small amounts of nuclear DNA or mtDNA are available for testing, researchers often utilize the polymerase chain reaction (PCR) to amplify specific regions of interest. PCR technology was developed in recent years and has been applied to a wide variety of studies, allowing researchers to obtain genetic information from very small portions of bodily tissue or fluid. Amplification of specific regions of the mitochondrial D-loop has been proven successful with both “universal” and species-specific primers. The process of PCR involves first denaturing the DNA template with heat in the presence of excess primers and the four bases (dNTPs). The reaction mixture is then cooled to allow annealing of the primers to their target sequence, followed by a period of raised temperature in which the attached primers are extended with *Taq* polymerase, which adds nucleotides at a rate of 200 per second at its optimum 75° C temperature. *Taq* polymerase is a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (Saiki et al., 1988) Each of the products from a single cycle serves as a template for the next cycle, so each successive round doubles

the total amount of target DNA within the reaction mixture.

It is possible to differentiate varying mtDNA types by combining PCR amplification of the hypervariable D-loop region with restriction digest analysis (Martin et al., 1992). This method is relatively rapid in comparison to traditional RFLP analysis, as it does not require as much sample quantity of mtDNA, and the mtDNA that is isolated need not be purified. It also avoids the need for any radioactivity to visualize DNA.

Composition of western Atlantic loggerhead stocks

The southeast coast of the United States is home to 35,000 reproductive adult female loggerheads, with 14,000 nesting annually (Murphy and Hopkins, 1984). Individual rookeries may span tens to hundreds of kilometers. While nesting has been recorded as far south as Texas and as far north as Virginia, aerial surveys indicate that 90% of these nests are concentrated in Florida, and the other 10% are clustered primarily in Georgia and South Carolina (Murphy and Hopkins-Murphy, 1989). Thus, there are two major rookeries in the southeastern United States (Florida and Georgia/South Carolina), each composed of hundreds of miles of nesting beaches. Slight morphological differences have been noted among turtles nesting in these two major areas (Stoneburner et al., 1980). Mitochondrial DNA analysis has determined that these geographically separated populations are genetically distinct, with low levels of maternal gene flow (Bowen et al., 1993a; Murphy and Hopkins-Murphy, 1989). Two haplotypes predominate in these loggerhead populations. Using Bowen et al (1993a) conventions, they are the B and D haplotypes. The southern (Florida) population is characterized by a

mixture of these haplotypes, with a frequency of 0.68 (D) and 0.32 (B) (n=28 individuals). The Georgia/South Carolina population is comprised exclusively of the B haplotype (n=60). The nucleotide sequence divergence of these two haplotypes is relatively deep, with a mean sequence divergence $p=0.8\%$ (Bowen et al., 1993a).

A recent study of a juvenile feeding population of loggerhead turtles in Charleston Harbor, South Carolina used mtDNA analysis to depict the population as a composite of natal populations from both the Florida and Georgia/South Carolina rookeries (Sears et al., 1995). Approximately 50% of the turtles comprising the Charleston Harbor population are derived from the northern Georgia/South Carolina rookery, a significantly greater proportion than would be expected if there was random selection of juvenile feeding locations.

Statement of problem and conservation relevance

The Chesapeake Bay contains a significant summer feeding population of juvenile loggerhead turtles. While the mortality rate of hatchling sea turtles and eggs is extremely high, this is offset in undisturbed populations by the large clutch size of nests. The mortality rate of adult and juvenile sea turtles is extremely low under natural conditions, but human interactions have led to increased mortality rates at all stages of the life cycle. Human activities are the direct cause of thousands of sea turtle deaths each year (National Research Council, 1990), and the northern (Georgia/South Carolina) population of loggerheads shows evidence of long-term decline (Richardson, 1982). The number of nesting females there is declining at the rate of about 4% per year (Musick, 1988).

Between 50 and 200 dead sea turtles (90% of which are loggerheads) strand on the beaches of the Chesapeake Bay each year, with over one-third of these deaths linked to human activity such as drowning in fishing nets and mutilation by boat propellers (Keinath et al., 1987). Due to the migratory nature of sea turtles, management practices must be tailored for each nesting population in order to effectively conserve genetic diversity. A population over-exploited by humans or other causes at one location will be unlikely to recover or be reestablished naturally, since natal homing prevents significant immigration of nesting females from other nesting beaches to the beach supplying the endangered population. Equally disturbing as the loss of populations themselves is the overall loss of species genetic variation under such circumstances, particularly if the affected population is one which is small and variant from the norm. Natal homing “mistakes” by adult females have been known to occur (LeBuff, 1974), but not in high enough frequency to replace other populations. Each rookery should be treated as an autonomous demographic and genetic entity (Bowen et al., 1993a). This study seeks to identify the natal origin of summer foraging loggerhead turtles in the Chesapeake Bay using analysis of mtDNA.

Materials and Methods

Sampling

Since loggerheads are a threatened species, it was not feasible to get large sample sizes in a juvenile population by sacrificing individuals. Instead, blood samples were drawn from individuals that were in the process of being tagged, weighed, measured, and released at the Virginia Institute of Marine Science (VIMS) turtle greenhouse located at the mouth of the York river in Gloucester Point, Virginia. All loggerhead samples were obtained through the VIMS sea turtle stranding network. This network includes state and federal agencies, volunteer organizations, and private citizens. Live turtles of varying carapace lengths (and thus varying ages) had approximately 5 ml of blood drawn from the dorsal cervical sinus (Figure 2) and stored in a lysis buffer (10 mM Tris, 100 mM EDTA, 0.5% SDS, pH 8). Blood samples were available from 63 individuals. Each sample was collected between May and July in the years 1989-1994 (Table 2). Samples collected from individuals from 1989 to 1993 were centrifuged briefly and stored at -20° C. Samples taken in 1994 were kept at 4° C. Heart tissue from two recently dead loggerheads was taken following necropsy in July 1994.

Preparation of a probe for Southern blotting

Southern blotting (Southern, 1975) is a technique used to visualize specific regions of DNA on a gel by hybridizing a probe to the DNA of interest. In an effort to obtain a probe which could be used to detect mtDNA against a total genomic DNA background, high quality mtDNA was isolated and purified from 24 separate 1g samples of heart tissue using protocols of Lansman et al. (1981). Intact mitochondria were separated from intact nuclei and cellular debris using differential centrifugation. Cesium chloride density-gradient centrifugation was utilized to purify closed circular mtDNA, by removing the more abundant nuclear DNA. This method takes advantage of the closed-circular supercoiled structure of mtDNA by using ethidium bromide to intercalate the bases of all the DNA present. Linear DNA is able to intercalate more ethidium bromide than supercoiled mtDNA, decreasing its density. The difference in densities cause these two forms of DNA to migrate to different positions when placed in a CsCl density gradient and spun at 70,000 rpm for 24 hours. Two distinct bands resulted, with the linear DNA band (containing both nuclear DNA and any nicked mtDNA) on the top, and the supercoiled mtDNA located just below. The mtDNA band was collected by tube puncture and bottom dripping.

The mtDNA fraction was cleaned by butanol extractions (butanol saturated with 5M NaCl), which removed ethidium bromide. The butanol and CsCl were removed from the mixture by serial dialysis. Dialysis tubing is semi-permeable, allowing ions and solutes to pass through, but not DNA. At 4° C, two 12 hour dialysis stages in 1X TE (10 mM Tris-Cl (pH 8.0); 0.5% SDS) were followed by two 12 hour stages in 0.1X TE.

The samples were then concentrated using ethanol precipitation. This procedure involved adding 1 μ l tRNA, 0.4x volume of 5M NaCl, and 2.2x volume of -20° C ethanol to 400 μ l aliquots. The mixture was shaken and frozen overnight at -20° C. It was then spun at 14,000 rpm for 20 minutes at 4°C, the supernatant decanted, and any remaining supernatant was removed under vacuum. The pellet was rehydrated in 0.1X TE.

Following ethanol precipitation and rehydration, mtDNA was cleaved by the restriction endonuclease *Eco* RV into four fragments: 7.4 kb, 4.65 kb, 3.8 kb, and 0.75 kb. Using a shotgun approach with a ratio of 1:3 vector to insert, the mixture was ligated into the plasmid vector Bluescript KS⁺ using the same restriction enzyme. *E. coli* transformant cells were grown on selective media. Successful clones contained functional genes coding for ampicillin resistance, but had incapacitated Lac Z genes (which produce β -galactosidase), thus preventing them from turning blue on a lactose rich substrate. Clones were screened against non-recombinant Bluescript KS⁺ to verify success.

Clones were grown in overnight cultures of LB medium supplemented with ampicillin and prepared in large quantities, and isolated using alkaline lysis (Sambrook et al., 1989). At this point the mtDNA clones were nick translated utilizing biotin-14-dATP as a label (BioNick Labeling System, BRL). A 50 μ l reaction containing 5 μ l of 10X dNTP mix (0.2 mM each dCTP, dGTP, dTTP; 0.1 mM dATP; 0.1 mM biotin-14-dATP; 500 mM Tris-HCl (pH 7.8); 50 mM MgCl₂; 100 mM β -mercaptoethanol; 100 mg/ml nuclease-free bovine serum albumin (BSA)), 1 μ g probe DNA diluted to 40 μ l, and 5 μ l of 10X enzyme mix (0.5 units/ μ l DNA polymerase I; 0.0075 units/ μ l Dnase I; 50 mM Tris-

HCl (pH 7.5); 5 mM magnesium acetate; 1 mM β -mercaptoethanol; 0.1 mM phenylmethylsulfonyl fluoride; 50% (v/v) glycerol; 100 μ g/ml nuclease-free BSA) was incubated 90 minutes at 15° C, and stopped with 5 μ l of 300 mM EDTA. In this process DNA was cut into small fragments, and adenine bases in the DNA molecules were replaced with biotin-labelled adenine bases. Unincorporated nucleotides were removed by size exclusion chromatography with a NICK column (Pharmacia Biotech). The resultant probe was stored at -20° C.

Total genomic DNA was isolated from blood using a modification of the methods of Blin and Stafford (1976). Approximately 0.25 μ l of blood was added to 130 μ l of a lysis buffer (10 mM Tris-Cl (pH 8.0); 0.1 M EDTA (pH 8.0); 0.5% SDS), manually chopped and ground, and then vortexed. It was then subjected to successive extractions with pure phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). Two-tenths volumes of 10 M ammonium acetate and 2 volumes of 95% ethanol at -20°C were added to the resulting mixture and stored at -20°C overnight. Following an ethanol precipitation, pellets were resuspended in 20 μ l 1X TE.

Total genomic DNA was digested with *Ava* II and *Stu* I, both informative restriction endonucleases capable of distinguishing between the B and D loggerhead haplotypes, as described by Bowen et al. (1993a). Digestion reactions contained 6 μ l of genomic DNA, 2 μ l of deionized water, 1 μ l appropriate buffer, and 4 units enzyme. All digests were incubated overnight at 37° C. Each digestion mixture was loaded to a 1% agarose gel, along with an appropriate size standard and positive control (3 μ l of probe loaded into a lane), and run at 80-100 volts for 2 hours.

A standard Southern blot hybridization protocol was used to visualize the DNA fragments, using the biotinylated clone of loggerhead mtDNA as probe (Sambrook et al., 1989). The gel was first depurinated for two fifteen minute shaking periods with 0.25 M HCl, which broke the DNA into smaller fragments for more efficient transfer onto a nylon support membrane. The DNA was then subjected to strong base conditions (1.5 M NaCl; 0.5 M NaOH) for two 20 minute washes, causing it to become single-stranded by breaking the hydrogen bonds holding the strands together. Two 20 minute washes in a neutralization buffer (1.0 M Sigma 7-9; 1.5 M NaCl; pH 8.0) were then used to stabilize the fragments.

DNA was transferred to a nylon support membrane using capillary transfer and a high salt buffer (10X SSC: 1.5 M NaCl; 0.15 M citric acid trisodium salt, dihydrate; pH 7.5) overnight. The membrane was cross-linked with ultraviolet light for 1 minute to bind the DNA to it. The filter was then incubated for two hours at 42 °C with a prehybridization mixture (2.5 ml formamide; 1.25 ml 20X SSC; 0.5 ml Dendhardt's solution; 0.25 ml 0.5 M NaPO₄, pH 6.5; 0.3 ml sterile water; 0.2 ml denatured iced calf thymus DNA) containing single-stranded calf thymus DNA, which prevented non-specific binding. The probe (0.5 µg) was made single stranded by boiling and icing for 10 minutes each, then added to the mixture and allowed to hybridize overnight.

A series of stringency washes followed hybridization, designed to reduce non-specific probe binding. The washes consisted of two three-minute rinses in wash 1 (2X SSC; 0.1% SDS), two three-minute rinses in wash 2 (0.2X SSC; 0.1% SDS), and two 15 minute rinses in wash 3 (0.16X SSC; 0.1% SDS), all at 42 °C. Stringency washes were

followed by a one minute wash in BluGene buffer 1 (0.1 M Tris-HCl (pH 7.5); 0.15 M NaCl), followed by a 1 hour incubation at 42° C with a blocking solution of 3% BSA made with BluGene buffer 1. After blocking, a solution of 7 ml BluGene buffer 1 and 7 µl streptavidin-alkaline phosphatase (1 mg/ml in 3 M NaCl; 1 mM MgCl₂; 0.1 mM ZnCl₂; 30 mM triethanolamine; pH 7.6) was added and incubated with the filter for 10 minutes at 42° C. This was followed by two 15 minute washes at 42° C in BluGene buffer 1. Then the filter was washed with BluGene buffer 3 (0.1 M Tris-HCl (pH 9.5); 0.1 M NaCl; 50 mM MgCl₂) for 10 minutes at 42° C. The filter was developed in a solution of 7.5 ml BluGene buffer 3, 33 µl NBT (75 mg/ml nitroblue tetrazolium in 70% dimethylformamide), and 25µl BCIP (50 mg/ml 5-bromo-4-chloro-3-indolylphosphate in dimethylformamide) at 42° C in the dark for approximately one-half hour. This procedure produced a filter that could be analyzed directly.

Amplification of the D-loop using the polymerase chain reaction

PCR was used to amplify the approximately 420 bp D-loop of loggerhead turtle mtDNA using oligonucleotide primers complementary to the 5' and 3' ends of the loggerhead D-loop (CR-1 and CR-2) as described by Norman et al. (1994). The sequence of these primers was:

CR-1: 5' TTG TAC ATC TAC TTA TTT ACC AC 3'

CR-2: 5' GTA CGT ACA AGT AAA ACT ACC GTA TGC C 3'

A Perkin-Elmer PCR kit was used to prepare 50 µl reaction mixes, each containing 1 µl of template (total genomic) DNA, 26.2 pmoles of primer CR-1, 28.2 pmoles of primer

CR-2, 5 μ l of buffer (100 mM Tris-HCl (pH 8.3); 500 mM KCl; 1.5 mM MgCl₂; 0.01% w/v gelatin), 1 μ l each of 10 mM dATP, dGTP, dCTP, and dTTP, and 1.25 units of *Taq* polymerase. Reaction conditions for amplification in a Perkin Elmer Cetus DNA Thermal Cycler were: 6 minutes at 94°C, followed by 40 cycles of 2 minutes at 94°C, 2 minutes at 50°C, and 4 minutes at 72°C. This was completed with a final 14 minute extension at 72°C, and then stored at 4°C. Each group of samples amplified contained a negative control (reaction mix without any template DNA) in order to detect contamination. PCR products (5 μ l) were screened on a 1% agarose gel containing a DNA mass ladder (BRL), stained with ethidium bromide, and visualized on a UV light table to determine if D-loop amplification was successful.

Sequence data from original electrophoretograms for the D-loop in the B and D haplotypes were obtained (Brian Bowen, University of Florida, personal communication, 1995) and input to the DOS compatible PC Gene software package (release 6.70, (C) A. Bairoch/University of Geneva/Switzerland/(TM) IntelliGenetics Inc. serial number IGI2981). These sequences are shown in Figure 3. A restriction-site analysis was performed for each sequence, generating a profile of cuts by known restriction endonucleases. From these lists, four enzymes (*Apo* I, *Hae* III, *Sau* 96I, and *Ssp* I) were chosen for their ability to distinguish between the different haplotypes (Table 3). *Apo* I is a 6 base-cutter, and the reaction mixture contained 7 μ l of PCR product, 1 μ l of BSA (100 ng/ml acetylated BSA), 1 μ l of buffer (100 mM NaCl; 50 mM HCl; 10 mM MgCl₂, 1.0 mM dithiothreitol), and 2 units of enzyme. This reaction mixture was incubated at 50°C for 18 hours. *Hae* III (a 4 base-cutter) digestion mixtures contained 8 μ l PCR

product, 1 μ l of buffer (50 mM NaCl; 10 mM Tris-HCl; 10 mM MgCl₂; 1.0 mM dithiothreitol), and 5 units of enzyme for 18 hours at 37° C. *Sau* 96I (a 5 base-cutter and isoschizomer of *Asu* I) digestion mixtures contained 8 μ l PCR product, 1 μ l of buffer (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1.0 M dithiothreitol), and 5 units of enzyme for 18 hours at 37° C. *Ssp* I (a 6 base-cutter) reaction mixtures contained 8 μ l of PCR product, 1 μ l of buffer (50 mM NaCl; 10 mM Tris-HCl; 10 mM MgCl₂; 1 mM dithiothreitol), and 2.5 units of enzyme. Each of the digests using this enzyme was incubated at 37°C for 18 hours.

Digestion products for each turtle sample were subjected to gel electrophoresis in a 2.5% agarose gel containing a 1 kb size standard (BRL). Gels were stained with ethidium bromide and viewed under UV light. As many as four restriction fragments of the approximately 420 base pair (bp) D-loop were visualized under these conditions and photographed with Polaroid 667 film.

RFLP analysis

Analysis of the D-loop restriction fragment data was done by comparing (with the aid of a size standard) the restriction digest patterns expected for each haplotype with the actual patterns produced in each of the samples. Haplotype frequencies were calculated from direct counts of individuals of each haplotype. Chi-square analysis was used to determine if these haplotype frequencies were significantly different from either of the source nesting populations, and to determine if the Chesapeake Bay population was comprised exclusively of one nesting population.

Results:

Southern blot hybridizations

Mitochondrial DNA fragments from total genomic DNA isolations were not successfully visualized on Southern blot filters. While both the biotinylated λ -*Hind* III ladder and the probe positive control were visualized (indicating effective Southern blotting technique and successful probe-probe hybridization, respectively), no bands were seen on lanes containing total genomic DNA digested with *Ava* II or *Stu* I. A Southern blot containing mtDNA purified from heart tissue and digested with *Eco* RV was hybridized in order to verify that the probe was capable of binding to loggerhead turtle mtDNA, and the resulting filter revealed the expected mtDNA fragments (Figure 4). Therefore it was concluded that there was insufficient mtDNA in the total genomic blood isolations to detect using the biotin labelling system.

RFLP analysis of D-loop amplifications

The D-loop region was successfully amplified with primers provided by Bowen (Norman et al., 1994). Only a single band in the desired region of 420 bp was detected after gel electrophoresis of all samples, and none of the negative controls showed any

products (Figure 5). Digestion of the PCR products with each enzyme yielded a restriction fragment pattern for each individual. Analysis of the 62 loggerhead samples revealed only two fragment patterns for each restriction enzyme (Figures 6-9).

Examination of the fragment patterns allowed straightforward determination of the B and D haplotypes. All of the 62 loggerhead blood samples were positively identified based on their D-loop restriction digest patterns using *Apo I*, *Hae III*, *Sau 96 I*, and *Ssp I* (Table 4). Each enzyme alone sufficiently discriminated between haplotypes B and D. All restriction patterns were consistent in identifying the haplotype for each sample, with the exception of one individual (sample 35). This sample deviated from the expected pattern for *Ssp I*, but upon examination of tagging records, was determined to be a Kemp's r rather than a loggerhead, and was not included in the analysis.

The samples from the Chesapeake Bay population of loggerheads were comprised of the two genotypes (B and D), with 69% designated as haplotype B (n=43), and 31% designated as haplotype D (n=19). Using chi-square analysis, the haplotype frequencies of the Bay population were determined to be significantly different from both the nesting population of Georgia/South Carolina ($X^2=44.93$, $df=1$, $p<0.001$) and of Florida ($X^2=64.0$, $df=1$, $p<0.001$). Such a significant difference in haplotypic frequencies suggested that the loggerhead population in the Chesapeake Bay is not recruited exclusively from either Georgia/South Carolina or Florida, but is instead a mixed stock. Evidence of a mixed stock led to two possibilities: that the Chesapeake Bay foraging population is drawn at random from both natal rookeries, or that juveniles from either the northern or southern rookery are preferentially recruited to the Bay. The possibility that

the Bay loggerhead population is a random mixing of individuals from along the coast of the southeast United States was tested by chi-square analysis. Since approximately 90% of nests in the southeast United States are located on Florida beaches (Murphy and Hopkins-Murphy, 1989), if random mixing of stocks occurs in the Chesapeake Bay, then one would expect 90% of the turtles in the Bay to have originated from Florida rookeries. This generated an expectation of 56 of the 62 turtles sampled to have come from Florida. In such a scenario, it would be expected that 61% (n=38) of the 62 samples would be haplotype D, and 39% (n=24) would be haplotype B. The variation between these expectations and the data generated in this study were significantly different ($X^2=24.54$, $df=1$, $p<0.001$). Therefore random mixing does not occur in the Chesapeake Bay juvenile population. Instead, juveniles from Georgia/South Carolina utilize the Bay as a foraging refuge significantly more frequently than their neighbors to the south.

Since the Chesapeake Bay contains a mixture of two haplotypes, and one of these (D) is found exclusively in the Florida rookery (Bowen et al., 1993a), the potential contribution of the Florida rookery to the Bay juvenile population could be solved with a single variable equation:

$$D_{CB}=D_F*X$$

In this equation, D_F is the frequency of haplotype D in Florida, D_{CB} is the frequency of haplotype D in the Chesapeake Bay, and X is the fraction of the Chesapeake Bay population that is recruited from Florida rookeries. The remainder (1- X) was assumed to be recruited from the Georgia/South Carolina rookery since these two locations contain roughly 99% of the known loggerhead nests in the northwestern Atlantic (Sears et al.,

1995). Of the turtles sampled, 46% were recruited from the Florida rookery, while the remaining 54% originated from the beaches of Georgia/South Carolina.

To determine if the relative contribution of the two rookeries was consistent from year to year, the Chesapeake Bay stock composition was analyzed for yearly variation in haplotypic frequencies (Table 5). There were no significant differences in each year's sample from the mean of the combined haplotype frequencies when subjected to chi-square analysis, indicating that these frequencies are relatively stable.

Discussion:

Restriction fragment length polymorphism analysis of the total loggerhead mitochondrial genome by means of Southern blotting with a biotin-labelled probe produced uninformative hybridization filters. The lack of mtDNA bands probably resulted because of the extremely low quantity of target mtDNA present in sea turtle blood. A previous study successfully employed hybridization and visualization of loggerhead mtDNA fragments from blood. Sears et al. (1995) used ³²P-labelled nucleotides and random priming to create a probe of higher sensitivity and greater specific activity. While it would have been possible to use these techniques for the Chesapeake Bay loggerheads, it was decided that this study would employ non-radioactive protocols to resolve the population structure.

Restriction enzyme analysis of the amplified D-loop region of loggerhead mtDNA was used to classify mtDNA haplotypes. Sequence data of the D-loop specific for the B and D haplotypes were provided by Brian Bowen (University of Florida, personal communication, 1995), and were used to choose four informative restriction endonucleases to distinguish between two haplotypes. These enzymes sorted the two haplotypes across all samples, and each enzyme supported the haplotype designation of the other three for each individual. After assigning each individual to a haplotype based

on D-loop RFLPs, these frequencies were compared to haplotype frequencies from the two natal rookeries (Bowen et al., 1993a), and the relative contributions of each to the Chesapeake Bay population was determined.

The Chesapeake Bay population of loggerhead turtles is composed of approximately equal contributions from two major nesting rookeries in the southeast United States. Only 10% of active loggerhead nesting takes place on Georgia/South Carolina, yet just more than half of the turtles sampled in the Bay were derived from this rookery location. It is likely that juveniles from Georgia/South Carolina preferentially choose Chesapeake Bay in their foraging site selection. This evidence concurs with Sears et al. (1995) stock assessment of 31 juveniles in Charleston Harbor, in which it was demonstrated that 50% of the population was derived from each of the Georgia/South Carolina and the Florida rookeries. It also supports heavy metal and epibiota studies suggesting that juvenile turtles hatched from more northern rookeries tend to stay along the coast of the southeastern United States, while those from more southern rookeries tend to forage in the more tropical regions of the Caribbean and the Gulf of Mexico (Caine, 1986; Stoneburner et al., 1980; Meylan et al., 1983; Richardson, 1982).

The determination of the source nesting populations contributing to the Chesapeake Bay loggerhead population is made under the assumption that there are only two such potential sources of the Chesapeake Bay population. There is evidence for loggerhead nesting as far north as Virginia Beach, where two nests hatched in 1994 (Musick, 1988), as well as some nesting activity in North Carolina, Texas, and Mexico. However, these nests constitute a combined estimate of 1% of the total number of nests in

the northwestern Atlantic arena, and thus are discounted from this study.

A more problematic potential source of juvenile loggerhead turtles are nesting beaches in the Mediterranean. There is a group of nesting populations approximately the size of the Georgia/South Carolina rookery located around Greece, Cyprus, and Turkey which is likely to be derived from the Florida population (Bowen et al., 1993a). There is also a significant juvenile foraging population located in the Mediterranean Sea, more than half of which is composed of loggerheads born in the western Atlantic (Lahiri et al., 1994). Because this juvenile population contains such a large percentage of western Atlantic turtles, but their presence is not evident on Mediterranean nesting grounds, it seems likely that juvenile loggerheads native to the western Atlantic are capable of transversing the ocean and returning back again to their natal origin to nest when sexual maturity is reached, even against the prevailing eastward current into the Mediterranean Sea. Hatchling turtles are not as capable of swimming against the current, however, and it seems reasonable that Mediterranean loggerheads may never reach currents such as the North-Atlantic gyre and circulate around as it appears that loggerheads from the western Atlantic do.

There are two major lines of evidence suggesting that Mediterranean loggerhead hatchlings do not circulate through the Atlantic. Firstly, researchers observe all size classes of loggerheads within the Mediterranean Sea, implying that they can complete the life cycle there (Groombridge, 1990). Secondly (and perhaps more significantly), the frequencies of mtDNA haplotypes in some eastern Atlantic juvenile foraging grounds do not vary significantly from haplotypic frequencies in the southeastern United States

(Brian Bowen, University of Florida, personal communication, 1995), suggesting that stocks in the open ocean are not “watered down” by Mediterranean gene frequencies. These data suggest that Mediterranean stocks of loggerheads do not contribute to western Atlantic populations.

Accumulating evidence that juvenile foraging grounds are typically composed of mixed stocks, and that not all sea turtles from the same stock travel together, suggests that there is no “programmed” migratory pathway designating life history patterns. This study raises more interesting questions that will be answered by future researchers. Why do loggerheads from northern nesting sites tend to select juvenile foraging havens along the east coast of the United States, and what mechanism controls how they go about choosing this site?

In this study between-year variation in haplotypic frequencies was large, but was determined not to be significant due to the small sample sizes for each year. Additionally, samples were available for only a few years, leaving long-term variation in question. Either a significantly larger sample size within years, or testing of similar sample sizes over many more years would be needed to determine if the Chesapeake Bay stock composition actually differs significantly from year to year. This would be an interesting question to test, since the migrational patterns of juveniles are still in question. Broader knowledge of the movements of this species at the juvenile, adult, and hatchling stages is critical for assessing the potential impact of humans on future populations of loggerheads in the north Atlantic.

This study demonstrates that the Georgia/South Carolina rookery, already in

decline, is disproportionately affected by natural and human-related activities which negatively affect the loggerhead population in the Chesapeake Bay. This is due to the northern rookery being an order of magnitude smaller than the Florida rookery. The Georgia/South Carolina rookery is also the location of two unique but uncommon haplotypes (A and C, see Bowen et al., 1993a), so this region is genetically distinctive. From a conservation genetics perspective, these unique haplotypes should be preserved. Therefore, the Chesapeake Bay loggerhead population should be monitored closely for activities detrimental to sea turtle survival. If there is a question of the long-term status of this species of sea turtle, it would behoove those making wildlife management decisions to be more liberal with intensive regulations on a population such as this than with an aggregate composed of turtles derived mainly from the less endangered Florida nesting population.

This assessment of the natal origin of juvenile turtles in the Chesapeake Bay allows for a better understanding of loggerhead life cycles and migratory pathways, and also identifies which nesting populations are impacted by human activities occurring in the Bay area, and vice versa. Such work has proven useful in the case of a juvenile Mediterranean population of loggerheads (Lahiri et al., 1994). It is estimated that up to 50,000 loggerheads are caught in driftnet fisheries in the region each year, and it was unknown which nesting population was being impacted by this toll. Mixed-stock analysis of mtDNA haplotypes determined that the level of contribution from the plentiful rookeries of the western Atlantic (southeastern United States) was roughly equal to the contribution by the more threatened Mediterranean rookeries. Thus, it was shown

that fishing practices in the Mediterranean have a more significantly negative impact on local nesting populations than on rookeries elsewhere in the world.

Valid information on juvenile stock composition allows wildlife managers to make informed decisions dealing with the interactions of humans and sea turtles. Without such information, governing agencies base their regulations only on the overall size of the populations they are dealing with, and neglect the fact that weaker stocks are being depleted. The 1982 U.N. Convention on the Law of the High Seas (Van Dyke, 1993), in concert with the 1983 U.N. Convention on Conservation of Migratory Species (Hykle, 1992), allow nations with nesting and developmental habitats for marine turtles to have jurisdiction over these animals on geographically remote feeding grounds, even if those feeding grounds are within the boundaries of another nation. Therefore, identifying the natal origin of sea turtles via genetic markers enhances the status of these threatened animals by providing a foundation for international agreements concerning their protection.

A variety of conservation practices having mixed success have been implemented for several species of sea turtles, ranging from protective legislation to headstarting (Pritchard, 1980). Successful long-term management of any existing population of these animals requires determination of their natal origin.

When combined with the long term decline in loggerheads in the Georgia/South Carolina nesting population, intense commercial fisheries already in existence in the Chesapeake Bay may be a substantial danger to the future success of loggerheads in the western Atlantic. Since the population in the Chesapeake Bay is made up largely of

loggerheads from the more endangered Georgia/South Carolina rookery, then wildlife management decisions should be enacted (and current protective measures maintained) to ensure the future success of this species.

Effective long term management of long lived species such as sea turtles requires knowledge of the genetic variation present in global and local populations, particularly where these populations may encounter the effects of human encroachment on native grounds. Equipped with knowledge of population structure, the application of mtDNA analysis is a powerful tool in learning about the migratory pathways of such species. This study used RFLP analysis of the mitochondrial D-loop to determine the mtDNA haplotype composition of loggerhead turtles feeding in the Chesapeake Bay, as well as determining the relative contribution of natal rookeries to this juvenile population. Combined with tagging, epibiota, heavy metal, and other mtDNA analyses by dedicated researchers throughout the loggerhead range, it provides a more complete picture of the life history of this threatened sea turtle.

Literature Cited

- Allard, M.W., M.M. Miyamoto, K.A. Bjorndal, A.B. Bolten, and B.W. Bowen. 1994. Support for Natal Homing in Green Turtles from Mitochondrial DNA Sequences. *Copeia* 1994:34-41.
- Anderson, S., A.T. Bankier, B.G. Barrell, M.H.L. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreiber, A.J.H. Smith, R. Staden, and I.G. Young. 1981. Sequence and organization of the human mitochondrial genome. *Nature* 290:457-465.
- Avise, J.C., B.W. Bowen, T. Lamb, A.B. Meylan, and E. Bermingham. 1992. Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Molec. Biol. Evol.* 9:457-473.
- Avise, J.C., C. Giblin-Davidson, J. Laerm, J.C. Patton, and R.A. Lansman. 1979. Mitochondrial DNA clones and matriarchal phylogeny within and among geographic populations of the pocket gopher, *Geomys pinetis*. *Proc. Natl. Acad. Sci. USA* 76:6694-6698.
- Avise, J.C., E. Bermingham, L.G. Kessler, and N.C. Saunders. 1984. Characterization of mitochondrial DNA variability in a hybrid swarm between subspecies of bluegill sunfish (*Lepomis macrochirus*). *Evolution* 38:931-941.
- Bjorndal, K.A., A.B. Meylan, and B.J. Turner. 1983. Sea turtle nesting at Melbourne Beach, Florida, I. Size, growth, and reproductive biology. *Biol. Cons.* 26:65-77.
- Blin, N. and D.W. Stafford. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.* 3:2303.
- Bowen, B.W. and J.C. Avise. 1995. Conservation genetics of marine turtles. In J.C. Avise and Hamrick, eds. Conservation Genetics: case histories from nature. Chapman and Hall, New York, in press.
- Bowen, B.W., J.C. Avise, J.I. Richardson, A.B. Meylan, D. Margaritoulis, and S.R. Hopkins-Murphy. 1993a. Population Structure of Loggerhead Turtles (*Caretta caretta*) in the Northwestern Atlantic Ocean and Mediterranean Sea. *Cons. Biol.*

7:834-844.

- Bowen, B.W., W.S. Nelson, and J.C. Avise. 1993b. A molecular phylogeny for marine turtles: Trait mapping, rate assessment, and conservation relevance. *Proc. Natl. Acad. Sci. USA* 90:5574-5577.
- Broderick, D., C. Moritz, J.D. Miller, M. Guinea, R.J. Prince, and C.J. Limpus. 1994. Genetic studies of the hawksbill turtle: evidence for multiple stocks and mixed feeding grounds in Australian waters. *Pac. Cons. Biol.* 1:123-131.
- Brown, W.M., M. George, Jr., and A.C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 76: 1967-1971.
- Buckley, H.A., L.R. Johnson, N.J. Shackleton, and R.A. Blow. 1982. Late glacial to recent cores from the eastern Mediterranean. *Deep Sea Research* 29:739-766.
- Caine, E.A. 1986. Carapace epibionts of nesting loggerhead sea turtles: Atlantic coast of U.S.A. *J. Exp. Marine Biol. Ecol.* 95:15-26.
- Caine, E.A. 1986. Carapace epibionts of nesting loggerhead sea turtles: Atlantic coast of USA. *J. Exp. Mar. Biol.* 95:15-26
- Carr, A. 1986. Rips, FADS, and little loggerheads. *BioScience* 36(2):92-100.
- Carr, A. 1967. *So Excellent a Fish: A Natural History of Sea Turtles*. Scribner, N.Y.
- Eckert, K.L. and S. A. Eckert. 1988. Pre-reproductive movements of leatherback sea turtles (*Dermochelys coriacea*) nesting in the Caribbean. *Copeia* 1988:400-406.
- Frazer, N.B. 1983. Survivorship of adult female loggerhead sea turtles, *Caretta caretta*, nesting on Little Cumberland Island, Georgia, U.S.A. *Herpetologica* 39(4):436-447.
- Groombridge, B. 1990. Marine turtles in the Mediterranean: distribution, population status, conservation. Report to the Council of Europe, Environment Conservation and Management Division, Nature and Environment Series 48. Strasbourg, France.
- Hallerman, E.M. and J.S. Beckman. 1988. DNA level polymorphism as a tool in fisheries science. *Can. J. Fish. Aquatic Sci.* 45.
- Hendrickson, J.R. 1958. The green sea turtle, *Chelonia mydas* (Linn.) In Malaya and Sarawak. *Proc. Zool. Soc. London* 130:455-535.

- Hillis, D.M. and Moritz, D. 1990. Isolation of Animal mtDNA using CsCl-PI Gradients. *Molecular Systematics* 278-284. Sinauer Associates, Inc., Sunderland, Massachusetts.
- Hykle, D.J. 1992. The migratory species (Bonn) convention and marine turtle conservation. Pages 61-63 in M. Salmon, and J. Wyneken, compiles, Proc. Eleventh Annual Workshop on Sea Turtle Biol. And Cons. NOAA Tech. Memo. NMFS-SEFC-302, National Technical Information Service, Springfield, VA.
- Keinath, J.A. 1994. Ph.D. dissertation.
- Keinath, J.A., Musick, J.A., and R.A. Byles. 1987. Aspects of the Biology of Virginia's Sea Turtles: 1979-1986. *Va. Jour. Sci.* 38(4): 329-336.
- Klinger, R.C. and J.A. Musick. 1994. Age and growth of loggerhead turtles which inhabit the Chesapeake Bay. *Copeia*, in press.
- Lahiri, S., B.W. Bowen, W.S. Grant, and R.J. Ferl. 1994. Application of mixed stock analysis to the demography of Atlantic and Mediterranean Loggerhead Turtles. Unpublished.
- Lansman, R.A., R.O. Shade, C.F. Shapira, and J.C. Avise. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. *J. Mol. Evol.* 17: 214-226.
- LeBuff, C.R. Jr. 1974. Unusual nesting relocation in the loggerhead turtle, *Caretta caretta*. *Herpetologica* 30:29-31.
- Limpus, C.J. 1979. Notes on growth rates of wild turtles. *Marine Turtle Newsl.* 10:3-5.
- Lutcavage, M. and J.A. Musick. 1985. Aspects of the biology of sea turtles in Virginia. *Copeia* 1985:449-456.
- Margaritoulis, D. 1988. Post-nesting movements of loggerhead sea turtles tagged in Greece. *Rapports de Commission Internationale de la Mer Mediterranee* 31(2):284.
- Martin, A.P, R.Humphreys, and S.R. Palumbi. 1992. Population genetic structure of the armorhead (*Pseudopentaceros wheeleri*) in the North Pacific Ocean: application of the polymerase chain reaction to fisheries problems. *Can. J. Fish. Aquat. Sci.* 49:2386-2391.
- Meylan, A.B., K.A. Bjorndal, and B.J. Turner. 1983. Sea turtle nesting at Melbourne

- Beach, Florida, II. Post-nesting movement of *Caretta caretta*. Biol. Cons. 26:79-90.
- McCoy, F.W. 1980. Climate changes in the eastern Mediterranean area during the past 240,000 years. Pages 79-100 in C. Doumas, editor. Thera and the Aegean World II: Papers and Proceedings of the Second International Scientific Congress, Santorini, Greece, August 1978. Thera and the Aegean World, London, England.
- Murphy, T.M., and S.R. Hopkins. 1984. Aerial and ground surveys of marine turtle nesting beaches in the southeast region, U.S. Report no. NA83-GA-C-00021. National Marine Fisheries Service, Southeast Fisheries Center Miami, Florida.
- Murphy, T.M., and S.R. Hopkins-Murphy. 1989. Sea turtle and shrimp fishing interactions: a summary and critique of relevant information. Center for Marine Conservation, Washington, D.C.
- Musick, J.A. 1988. The Sea Turtles of Virginia. Sea Grant Communications Office, Virginia Institute of Marine Science, Gloucester Point, VA.
- National Research Council. 1990. Decline of sea turtles: Causes and prevention. National Academy Press. Washington, D.C.
- Norman, J.A., C. Moritz, and C.J. Limpus. 1994. Mitochondrial DNA control region polymorphisms: genetic markers for ecological studies of marine turtles. Mol. Ecol. 3:363:374.
- Owens, D.W., M.A. Grassman, and J.R. Hendrickson. 1982. The imprinting hypothesis and sea turtle reproduction. Herpetologica 38:124-135.
- Pritchard, P.C.H. 1980. The Conservation of Sea Turtles: Practices and Problems. Amer. Zool. 20:609-617.
- Richardson, J.I. 1982. A population model for adult female loggerhead sea turtles (*Caretta caretta*) nesting in Georgia. Ph.D. Dissertation, Univ. Of Georgia, Athens, Georgia.
- Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487.
- Sambrook, J., E.F. Fritsch, T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press.

- Sears, C.J., B.W. Bowen, R.W. Chapman, S.B. Galloway, S.R. Hopkins-Murphy, and C.M. Woodley. 1995. Demographic composition of the juvenile loggerhead sea turtle (*Caretta caretta*) feeding population off Charleston, South Carolina: evidence from mitochondrial DNA markers. *Marine Biol.*, in press.
- Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
- Standora, E.A. and J.R. Spotila. 1985. Temperature dependent sex determination in sea turtles. *Copeia* 1985:711-722.
- Stoneburner, D.L., M.N. Nicora, and E.R. Blood. 1980. Heavy metals in loggerhead sea turtle eggs (*Caretta caretta*): Evidence to support the hypothesis that demes exist in the western Atlantic population. *J. Herp.* 14(2):171-175.
- Van Dyke, J.M. 1993. International governance and stewardship of the high seas and its resources. Pages 13-20 in J.M. Van Dyke, D. Zaelke, and G. Hewison, eds. Freedom for the Seas in the 21st Century: Ocean Governance and Environmental Harmony. Island Press, Washington D.C.
- Wilson, A.C., R.L. Cann, S.M. Carr, M. George, Jr., U.B. Gyllensten, K.M. Helm-Bychowski, R.G. Higuchi, S.R. Palumbi, E.M. Prager, R.D. Sage, M. Stoneking. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol. J. Linn. Soc.* 26:375-400.
- Yokenawa, H., K. Moriwake, O. Gotch, J. Hayashi, J. Watanabe, N. Miyashita, M. Petras, and Y. Tagashire. 1981. Evolutionary relationships among five subspecies of *Mus musculus* based on restriction enzyme cleavage patterns of mitochondrial DNA. *Genetics* 98:801-816.
- Zug, G.R., A.H. Wynn, and C. Ruckdeschel. 1986. Age determination of loggerhead sea turtles, *Caretta caretta*, by incremental growth marks in the skeleton. *Smithsonian Contrib. Zool.* 427:1-34.

Table 1: Current taxonomy of sea turtles	
Family Cheloniidae	<i>Chelonia mydas</i> (green)*
	<i>Caretta caretta</i> (loggerhead)*
	<i>Eretmochelys imbricata</i> (hawksbill)*
	<i>Lepidochelys kemp</i> (Kemp's Ridley)*
	<i>Lepidochelys olivacea</i> (olive ridley)
	<i>Natator depressus</i> (flatback)
Family Dermochelyidae	<i>Dermochelys coriacea</i> (leatherback)*

*These species occur in the Chesapeake Bay in Virginia

Table 2: Blood samples collected for analysis				
Turtle	Tag number	Carapace Length (cm)	Year	Location
1	SSB-801	55.7	1994	Potomac River Mouth
2	QQM-797	67.3	1994	Potomac River Mouth
3	SSB-883	58.8	1994	Potomac River Mouth
4	SSB-872	57.2	1994	Potomac River Mouth
5	SSB-845	57.4	1994	Potomac River Mouth
6	SSB-886	44.5	1994	Potomac River Mouth
7	SSB-860	56.6	1994	Potomac River Mouth
8	SSB-880	54.6	1994	Potomac River Mouth
9	QQZ-451	58.6	1994	Potomac River Mouth
10	SSB-877	53.6	1994	Potomac River Mouth
11	QQB-416	51.3	1990	Northhampton, VA
12	QQB-412	80.8	1990	Potomac River Mouth
13	QQB-486	68.2	1990	Hampton, VA Buckroe Beach Fishing Pier
14	QQB-476	62.3	1990	Potomac River Mouth
15	QQB-497	54.6	1990	Potomac River Mouth

Table 2 (continued)				
16	QQB-435	54.1	1990	Potomac River Mouth
17	QQB-488	62.5	1990	Mathews, VA Gwynns Island
18	QQB-433	52.2	1990	Potomac River Mouth
19	QQB-367	52.7	1990	Potomac River Mouth
20	QQB-439	47.7	1990	Hampton, VA Buckroe Beach Fishing Pier
21	QQB-319	54.0	1989	Potomac River Mouth
22	PPX-834	57.4	1989	Potomac River Mouth
23	PPX-853	77.4	1989	Potomac River Mouth
24	QQB-309	68.5	1989	Potomac River Mouth
25	PPX-855	58.0	1989	Potomac River Mouth
26	PPX-748	59.2	1990	Potomac River Mouth
27	PPX-851	62.8	1989	Potomac River Mouth
28	QQB-449	52.0	1990	Mathews, VA Gwynn's Island
29	QQB-437	47.0	1990	Potomac River Mouth
30	QQB-478	94.8	1990	Potomac River Mouth
31	QQB-424	64.6	1990	Potomac River Mouth

Table 2 (continued)				
32	PPX-888	53.0	1990	Potomac River Mouth
33	QQB-494	76.5	1990	Mathews, VA Gwynn's Island
34	QQB-480	68.8	1990	Potomac River Mouth
36	PPN-131	59.2	1989	Potomac River Mouth
37	QQB-322	63.3	1989	Potomac River Mouth
38	PPN-242	84.5	1989	Potomac River Mouth
39	QQB-376	56.4	1989	Potomac River Mouth
40	QQB-311	81.6	1989	Potomac River Mouth
41	QQB-378	59.0	1989	Hampton, VA Buckroe Beach Fishing Pier
42	PPX-743	87.0	1989	Potomac River Mouth
43	PPN-222	55.5	1989	Potomac River Mouth
44	PPX-843	67.5	1989	Potomac River Mouth
45	PPX-871	71.9	1989	Potomac River Mouth
46	SSB-915	58.8	1994	Potomac River Mouth
47	SSB-899	60.5	1994	Potomac River Mouth
48	SS B-805	44.9	1994	Potomac River Mouth

Table 2 (continued)				
49	SSB-898	57.8	1994	Potomac River Mouth
50	SSB-862	54.7	1994	Potomac River Mouth
51	SSB-837	56.0	1994	Potomac River Mouth
52	QQZ-364	55.4	1993	Potomac River Mouth
53	QQM-764	61.2	1993	Potomac River Mouth
54	QQZ-496	54.4	1993	Potomac River Mouth
55	QQZ-360	52.8	1993	Potomac River Mouth
56	QQZ-500	51.8	1993	Potomac River Mouth
57	QQZ-362	58.3	1993	Potomac River Mouth
58	QQZ-425	50.3	1993	Potomac River Mouth
59	QQZ-366	72.5	1993	Potomac River Mouth
60	WWX-354	62.0	1993	Potomac River Mouth
61	SSB-854	50.7	1994	Potomac River Mouth
62	QQZ-368	73.2	1993	Potomac River Mouth
63	SSB-827	51.2	1994	Potomac River Mouth
64	SSB-857	74.6	1994	Potomac River Mouth

Table 3: Restriction fragment patterns of the loggerhead D-loop resulting from cuts with diagnostic enzymes			
		Resulting Fragment Sizes (bp)	
Restriction Enzyme	Recognition Sequence	Haplotype B*	Haplotype D**
Apo I	5'...Pu ▾ A A T T Py...3'	346, 67	418
Hae III	5'...G G ▾ C C...3'	264, 61, 50, 38	301, 61, 56
Sau 96I	5'...G ▾ G N C C...3'	263, 150	418
Ssp I	5'...A A T ▾ A T T...3'	413	248, 170

*Haplotype B turtles have a D-loop length of 413 bp

**Haplotype D turtles have a D-loop length of 418 bp

Table 4: Loggerhead samples identified by haplotype

Turtle	Haplotype	Turtle	Haplotype	Turtle	Haplotype	Turtle	Haplotype
1	B	17	B	33	B	49	B
2	B	18	B	34	D	50	D
3	D	19	B	35	**	51	B
4	B	20	B	36	D	52	D
5	B	21	B	37	D	53	B
6	B	22	D	38	B	54	D
7	D	23	B	39	B	55	B
8	B	24	D	40	B	56	B
9	D	25	B	41	D	57	D
10	B	26	B	42	B	58	D
11	B	27	B	43	B	59	B
12	B	28	*	44	B	60	B
13	B	29	B	45	B	61	D
14	B	30	B	46	D	62	B
15	B	31	B	47	D	63	D
16	D	32	B	48	B	64	B

Total number of B haplotypes: 43 (69%)

Total number of D haplotypes: 19 (31%)

*quantity of DNA in digestions too low to visualize

**this sample was a Kemp's Ridley turtle

Table 5: Number of individuals of each haplotype collected by year					
Year	Haplotype B		Haplotype D		Chi square analysis: variation from the mean
1989	11	(69%)	5	(31%)	$X^2=0$, $df=1$, $p>.999$
1990	15	(88%)	2	(12%)	$X^2=2.55$, $df=1$, $p>0.1$
1993	6	(60%)	4	(40%)	$X^2=0.476$, $df=1$, $p>0.1$
1994	11	(58%)	8	(42%)	$X^2=0.974$, $df=1$, $p>0.1$
Mean	10.75	(69%)	4.75	(31%)	

Figure 1: Potential trans-Atlantic routes of hatchling loggerheads (Carr, 1986)

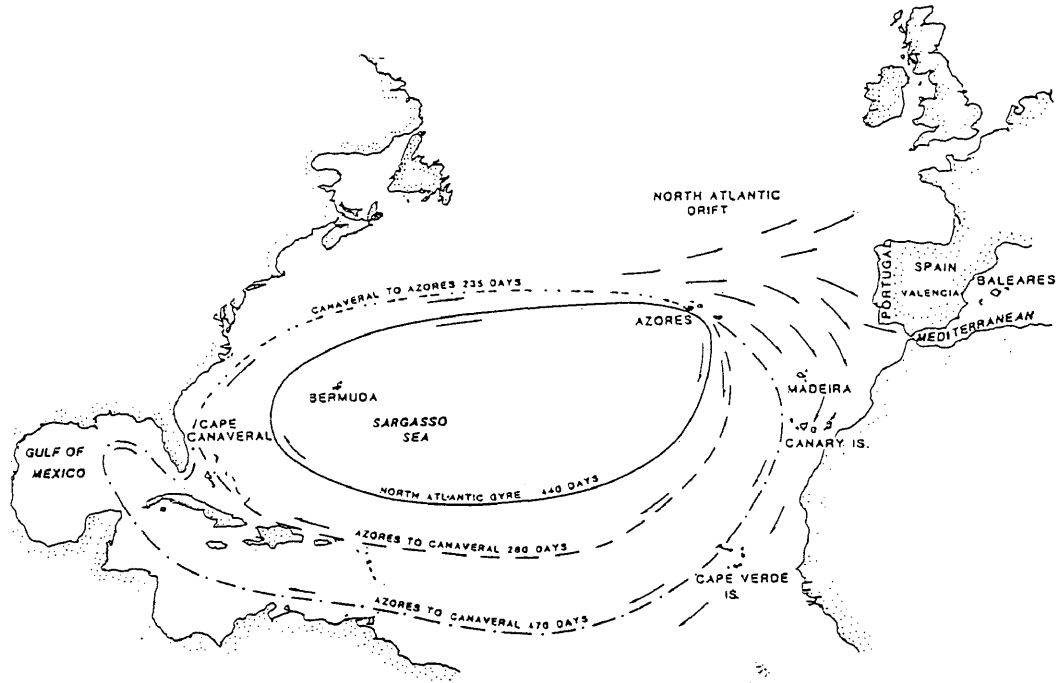


Figure 2: Physical location where blood was drawn from juvenile loggerheads

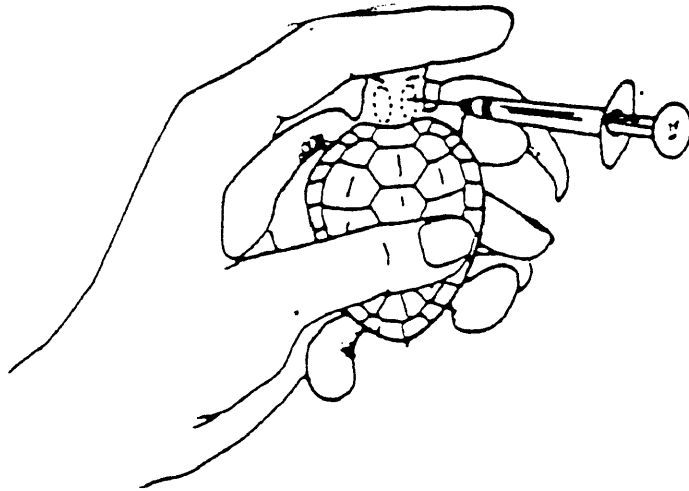


Figure 3: Sequence of the loggerhead mitochondrial D-loop for "B" and "D" haplotypes, with restriction sites of each of the four informative restriction endonucleases

B 5' C TACTT ATTTACCACT AGCATATGAT CAGTAATGTT GTCGATTAAT
 D 5' C TACTT ATTTACCACT AGCATATGAT CAGTAATGTT GTCGATTAAT

Apo I

↓

B TTGGCTTTAA ACATAA**AAAT** TTATTAATTT TACATAAACT GTTTTAGTTA
 D CTGACCTTAA ACATAAAAAC T-ATTAATTT TGCATAAACT GTTTTAGTTA

B CATGACTATT ATACAGGTAA TAAGAATGAA ATGATATAGG ACATAAAATT
 D CATGACTATT ATACAGGTAA TAGGAATGAA ATGATATAGG ACATAAAATT

B AAACCATTAT TCTCAACCAT GAATATCGTC GCAGTAATAG GTTATTTCTT
 D AAACCATTAT TCTCAACCAT GAATATCGTT ACAGTAATAG GTTATTTCTT

B AGTTCAGCTC ATCACGAGAA ATAAGCAACC CTTGTTAGTA AGATAACA
 D AGTTCAGCTC ATCACGAGAA ATAAGCAATC CTTGTTAGTA AGATA**CAATA**

↑

Ssp I

Figure 3 (continued):

Sau 96I *Hae* III
↓ ↓

B TTACCAGTTT CAGGCCATT AAGTCATATC GTACATAACT GATCTATTCT
D TTACCAGTTT CAAGTCCATT AAGTCATGTC GTACATAACT GATCTATTCT

Hae III
↓

B GGCCTCTGGT TG-TTTTTTC AGGCACATTA AGATAATAAA GTTCACTCGT
D GGCCTCTGGT TGGTTTTTTC AGGCACATTA AGGCAGTAA- GTTCATTTCGT

↑
Hae III

Hae III
↓

B TCCTCTTTAA AAGGCCTCTG GTT-----A AATGAGTTCT ATACATTAAA
D TCCTCTTTAA AAGGCCTCTG GTTGCAAGTA AATGAGTTCT ATACATTAAA

↑
Hae III

B TTTATAACCT GGCATACG 3'
D TTTATAACCT GGCATACG 3'

Figure 4: Southern blot hybridization filter containing three loggerhead mtDNA fragments (7.4 kb, 4.65 kb, and 3.8 kb) isolated from heart tissue after digestion with *Eco* RV (each fragment run in a separate lane on the left side of the filter). The lane at the far right is a λ -*Hind* III ladder, and next to it is the probe positive control.

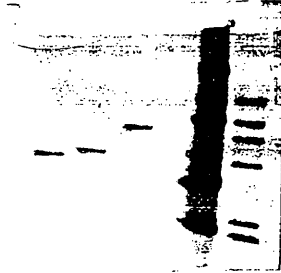


Figure 5: PCR product gel showing amplified loggerhead D-loop in the region of 420 bp. In the far left lanes is a DNA mass ladder, and in the lower right lane is the negative control.



Figure 6: Loggerhead D-loop fragment patterns resulting from digestion with *Apo* I (far left lane contains 1kb ladder)



Figure 7: Loggerhead D-loop fragment patterns resulting from digestion with *Hae* III (far left lane contains 1kb-ladder)

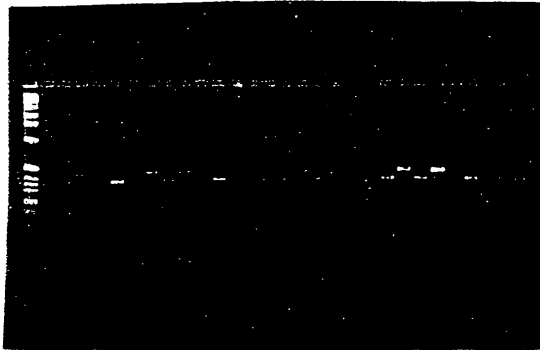


Figure 8: Loggerhead D-loop fragment patterns resulting from digestion with *Sau* 96I (far left lane contains 1kb ladder)

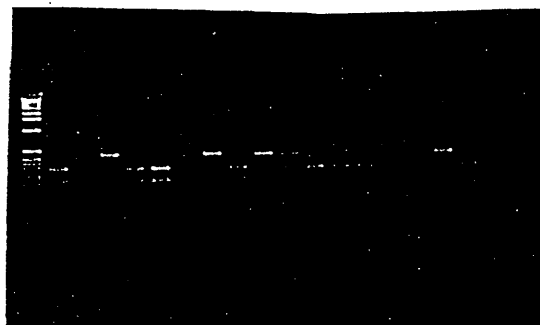


Figure 9: Loggerhead D-loop fragment patterns resulting from digestion with *Ssp* I (far left lane contains 1kb ladder)



VITA

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Born in St. Paul, Minnesota, January 9, 1971. Graduated from Thomas Jefferson High School in Alexandria, VA, June 1989, College of William and Mary, 1993. M.A. candidate, College of William and Mary, 1995, with a concentration in Biology, for which this thesis is a partial requirement. Married to Karen Gail Jones, William and Mary '93.