Copeia, 1998(4), pp. 1064–1069

Origin of Green Turtles, *Chelonia mydas*, at "Sleeping Rocks" off the Northeast Coast of Nicaragua

ANNA L. BASS, CYNTHIA J. LAGUEUX, AND BRIAN W. BOWEN

The largest foraging population of the Atlantic green turtle, *Chelonia mydas*, occurs in offshore waters of Caribbean Nicaragua (Carr et al., 1978). Green turtles are primarily herbivorous, and the extensive sea grass beds in Nicaraguan waters are utilized both by adults and juveniles (Nietschmann, 1972; Mortimer, 1981; C. Campbell, unpubl. data). Based on tags recovered from adult females, at least two nesting populations contribute individuals to this feeding aggregate: Tortuguero, Costa Rica; and Aves Island, Venezuela (Carr et al., 1978; Sole, 1994).

The largest nesting population of Atlantic green turtles is at Tortuguero, which supports an estimated annual nesting of approximately 14,000 females. The second largest nesting colonies in the Caribbean are the Surinam and Aves Island nesting colonies, which total approximately 2400 females annually (Ogren, 1989). The Tortuguero rookery has been the focus of long-term mark-recapture studies. From 1955 to mid-1977, 86.2% of international recoveries of tags placed on nesting females at Tortuguero came from the coastal waters of Nicaragua, prompting Carr et al. (1978) to suggest that this area is the principal foraging ground for the Costa Rican nesting colony. However, use of tag recoveries to identify stock composition of a foraging population can be biased by demographic factors, tag loss, and most especially uneven tagging and recapture efforts. Tagging studies are labor intensive and may yield only 1–8% tag recovery rates away from the nesting beach (Meylan, 1982; Limpus et al., 1992). Finally, most nesting areas are not subject to long-term tagging studies. For these reasons, relative contributions of individual nesting colonies to Caribbean feeding grounds are unknown.

An alternative approach, unbiased by factors related to tagging, uses mitochondrial DNA (mtDNA) markers to determine composition of the foraging population (Broderick et al., 1994). This approach, known as mixed stock analysis (Pella and Milner, 1987), depends on strong mtDNA haplotype frequency differences between nesting colonies to resolve relative contributions to a feeding aggregate. Mixed stock analysis has been applied to several species of marine turtle (Bowen et al., 1996; Bolten et al., 1998; Lahanas et al., 1998) but not to adult green turtles.

Historically, turtlers from the Cayman Islands and Nicaragua have utilized offshore waters of Nicaragua as a reliable source of green turtle meat (Parsons, 1962; Nietschmann, 1972). Turtlers believe that green turtles return at night to reefs or coral outcroppings (referred to as "sleeping rocks") after foraging on grass beds throughout the day, and the turtlers set their nets over these reefs or outcrops. The turtles are entangled during the night when they surface to breathe. Recent research has documented a harvest of at least 10,000 large juvenile and adult green turtles annually for personal consumption and sale in domestic markets (CJL, unpubl. data).

One goal of this study is to determine the origin for adult turtles off the northeastern coast of Nicaragua by utilizing mtDNA markers observed at eight Atlantic nesting colonies and one Mediterranean nesting colony (Encalada et al., 1996; Lahanas et al., 1998). Stock information has immediate applications; wildlife managers need to know which nesting populations occur in Nicaragua's offshore waters to assess impact of the harvest. A second goal is to compare haplotype frequencies between male and female feeding cohorts. Because only females come ashore to nest (hence only one sex is tagged at the nesting colonies), male migratory behaviors are not well known (but see Limpus et al., 1992; Ross and Lagueux, 1993; Fitz-Simmons et al., 1997). Genetic markers provide an opportunity to examine movements of both sexes and to test for possible differences in migratory behaviors.

MATERIALS AND METHODS

A 1-mL sample of whole blood, collected from each of 60 green turtles landed at Puerto Cabezas, Nicaragua, was mixed with 9 mL of lysis buffer (100 mM Tris-HCl, 100 mM EDTA, 10 mM NaCl, 1% SDS; pH 8.0) and stored at room temperature. Blood samples were collected in 1994, from March to May (n = 56) and in October (n = 4). Sampling periods were intended to occur outside the nesting season at Tortuguero (Carr et al., 1978). All turtles included in the analysis were harvested off the northeast

coast of Nicaragua. Animals were measured and sex confirmed when they were butchered for sale in local markets. To restrict the analysis to adult turtles, samples were collected only from animals in larger size classes: 30 females ranging in size from 88.3–105.7 cm straight-line carapace length (STCL) and 30 males (89.4–98.6 cm STCL). We assume that carapace length reflects maturity of an individual although this assumption may not hold in all cases, because a minority of turtles delay maturation until larger sizes (Carr and Goodman, 1970).

DNA isolations were conducted using standard phenol/chloroform methodology (Hillis et al., 1996). MtDNA control region fragments (510 bps in length) were amplified with a polymerase chain reaction (PCR) by using biotinylated versions of primers LTCM1 and HDCM1 (Allard et al., 1994). An 18-base "universal" M13 sequence was added to the 5′ end of primers to facilitate automated sequencing. Cycling thermal parameters used were as follows: one cycle at 94 C (3 min) followed by 35 cycles at 94 C (1 min), 50 C (1 min), and 72 C (1 min). Standard precautions, including negative controls (template-free PCR reactions), were used to test for contamination and to assure fidelity of PCR reactions (Innis et al., 1990).

Streptavidin-coated magnetic beads (Dynabeads M280 streptavidin, Dynal, Sweden) were used to purify PCR products (Mitchell and Merrill, 1989). Single-stranded templates were generated by denaturing magnetically captured double-stranded DNA with fresh 0.2 N NaOH and using the released (nonbiotinylated) strand for sequencing reactions. Single-stranded sequencing reactions were conducted with fluorescent-labeled M13 primers in a robotic work station (Applied Biosystems model 800). Sequencing products were analyzed with an automated DNA sequencer (Applied Biosystems model 373A) in the DNA Sequencing Core at the University of Florida.

Sequences were aligned using the overlap option in SeqEd (vers. 1.0.3). Misalignments were corrected by visual inspection and removal of unnecessary gaps. Polymorphic sites were identified within the sample of 60 individuals and then compared with polymorphic sites identified at nesting locations (n = 194) by Encalada et al. (1996) and Lahanas et al. (1998). Sequences that matched known haplotypes were collated for analysis. Unique haplotypes were resequenced to assure accuracy of haplotype designations.

Homogeneity of haplotype frequencies between rookeries and the foraging ground and between male and female cohorts was assessed using the program CHIRXC; probabilities were generated using Monte Carlo randomization (Roff and Bentzen, 1989; Zaykin and Pudovkin, 1993). The sequential Bonferroni technique was used to correct for simultaneous tests (Rice, 1989).

Relative contributions of surveyed Atlantic-Mediterranean green turtle rookeries [United States, Mexico, Costa Rica, Surinam, Brazil, Cyprus, Aves Island (Venezuela), Ascension Island (United Kingdom), and Guinea Bissau; see Encalada et al. (1996) for location information] were estimated using unconditional maximum-likelihood (ML) as implemented in the program UCON (M. Masuda, S. Nelson, J. Pella, User manual for GIRLSEM, GIRLSYM, and CONSQRT, US-Canada Salmon Program, 1991, unpubl.). This program uses an iterative algorithm to estimate most likely contributors to a mixed population based on haplotype frequencies in source populations (nesting colonies) and a mixed population (foraging ground). Standard deviations were generated using the infinitesimal jackknife procedure in UCON. Chapman (1996) suggests two potential null hypotheses for mixed stock analysis studies: (1) contributions to the mixed population are proportional to the size of the source population; and (2) contributions are equivalent from all source populations. The second null hypothesis was used as a starting point for iterations in maximum-likelihood analysis. Hypotheses concerning the proportion of contributions to the foraging area were tested using adjusted χ^2 goodness-of-fit tests (Sokal and Rohlf, 1981).

In the original survey of green turtle nesting locations by Encalada et al. (1996), haplotype frequencies of nesting populations at Aves Island (Venezuela) and Matapica (Surinam) did not differ significantly from each other as determined by a *G*-test of independence. Additional samples from Aves Island support the initial findings of Encalada et al. (1996); therefore, these populations were pooled into a regional population unit for mixed stock analysis (Lahanas et al., 1998).

Results

Two common Caribbean haplotypes, CM III and CM V, were identified in the 60 samples from the Nicaraguan foraging ground (appendix). Haplotype CM III was present in 54 individuals; the remaining six had haplotype CM V. Following Bonferroni corrections, the foraging ground population differed significantly ($P < 0.05$) from all nesting locations. An estimated 90% of individuals sampled off the northeast-

TABLE 1. MAXIMUM-LIKELIHOOD ESTIMATES OF CONTRIBUTION (± SD) OF *Chelonia mydas* NESTING LOCATIONS TO THE NORTHEASTERN COAST OF NICARAGUA.

	Tortuguero, Costa Rica	Aves Island/ Surinam
Females		
(n = 30)	0.964 (± 0.035)	0.036 (± 0.035)
Males		
(n = 30)	0.821 (± 0.073)	0.179 (± 0.073)
Overall		
(n = 60)	0.911 (± 0.038)	0.089 (± 0.038)

ern coast of Nicaragua originated at Tortuguero, Costa Rica; the remainder were attributed to the combined nesting populations at Aves Island (Venezuela) and Surinam (Table 1). No other nesting locations surveyed in the Atlantic contributed at detectable levels. The hypothesis that nesting beaches contribute equally to the foraging ground was rejected ($\chi_{adj}^2 = 40.01$; $P < 0.001$), but the hypothesis of contributions being proportional to the size of the nesting location could not be rejected ($\chi_{adj}^2 = 1.601$; $P > 0.05$).

Within the female sample, 29 were haplotype CM III and one was haplotype CM V. Within the male sample, 25 were haplotype CM III and five were haplotype CM V. These haplotype frequencies did not differ significantly ($\chi^2 = 2.962$; $P = 0.08$). Maximum-likelihood (ML) analysis of females attributed 96% (± 3%) of the total contribution to the Tortuguero nesting location, with the remaining 4% (± 3%) allocated to the Aves Island/Surinam nesting group; ML analysis of males attributed 82% (± 7%) to the Costa Rican nesting population and 18% (± 7%) to the Aves Island/Surinam nesting group (Table 1).

DISCUSSION

Tortuguero hosts the largest green turtle nesting aggregate in the Caribbean (Carr, 1967), with perhaps 90% of nesting in this region. Carr et al. (1978) hypothesized that the majority of Tortuguero nesting females forage off the Nicaraguan coast. The Tortuguero nesting population, however, is intensively studied, invoking concern that the estimated high contribution to the foraging ground is a result (in part) of a higher tagging effort on this beach. Furthermore, the conclusions of Carr et al. (1978), based on tags applied to nesting females, cannot be extended to males. MtDNA data provide an independent assessment of the composition of the Nicaraguan foraging stock (including both sexes). It appears that nesting locations do not contribute equally to foraging ground locations. Population size has been hypothesized to play a larger role in determining the contributions at foraging sites (Lahanas et al., 1998), and our results are consistent with expectations based upon the relative size of the nesting colonies at Tortuguero and Aves Island/Surinam. However, other factors that are not testable with these data may play a role in determining the composition of regional foraging grounds.

Although primary contributions appear to be from nesting aggregations at Tortuguero and Aves Island/Surinam, small contributions from other nesting aggregations in the western Caribbean (including those in Mexico and the United States) cannot be ruled out based on a sample of 60 individuals. However, the absence of "endemic" haplotypes from Mexican and U.S. nesting aggregates (Encalada et al., 1996) in the Nicaraguan foraging ground sample diminish the possibility of large contributions from these nesting locations. Increased sample sizes from the green turtle harvest in Nicaragua may provide better resolution and indicate whether other nesting colonies contribute individuals to this foraging area. More extensive sampling, including southeastern areas of coastal Nicaragua, could determine whether different nesting populations are partitioned among foraging habitats. Finally, a sampling regime that accounted for temporal differences is necessary for testing hypotheses concerning stock composition within and among years.

Although sample sizes of males (n = 30) and females (n = 30) were moderate, there was an indication of differences between rookery origins of male and female cohorts. Although there was only a marginal difference between male and female haplotype frequencies ($P = 0.08$), maximum-likelihood analysis indicated a greater, but not significantly different (paired $T = 5.49$, $P = 0.11$), contribution by the Aves Island/Surinam nesting group to the male foraging population (18%, males vs 4%, females; Table 1). The difference in estimated contributions to male and female cohorts could be an artifact of sample size. However, if this difference is supported by additional sampling efforts, then at least two general explanations could be advanced. First, differences in male and female recruitment to foraging grounds could be attributed to gender-specific variation in migratory behavior. This could explain higher male contribution from the more distant of the two contributing nesting groups. Second, this finding could be attributed to differences in sex ratios of breeding aggregates.

Lahanas et al. (1998) concluded from mtDNA data that Tortuguero and Aves Island/Surinam (believed to be the largest nesting colonies in the Caribbean) are the two primary sources of juvenile green turtles feeding in the Bahamas. Based on these findings, Lahanas et al. (1998) hypothesized a life-history model of random recruitment from regional nesting colonies to juvenile foraging grounds. The genetic composition of the Nicaraguan foraging ground (represented by the adults surveyed in this study) did not differ significantly from that of the juvenile foraging ground in the Bahamas (results not shown). Hence our results are consistent with the hypothesis that contributions of nesting colonies are proportional to the size of the rookeries, at least on a geographic scale which encompasses the greater Caribbean region.

These data add to the growing body of information indicating that molecular markers are a valuable tool for resolving migratory behavior of sea turtles. In weighing relative benefits of molecular and mechanical tags, two points bear consideration. First, results of a molecular study can be obtained in less time than results from tagging studies. Second, mechanical tags are irreplaceable for estimating parameters such as fecundity, age and growth, survivorship, as well as overall population trends. In this study, molecular data corroborated and extended findings based on long-term tagging programs. However, in the absence of field studies, genetic data alone may provide information urgently needed to manage and conserve these long-lived, migratory animals.

MtDNA data presented here are relevant to conservation studies and management programs, because wildlife managers need to know which nesting locations are impacted by the Nicaraguan harvest. In this case, nesting colonies in Costa Rica, Venezuela, and Surinam are linked to the fishery. We suspect that representatives of smaller nesting colonies are present in the harvest as well but eluded detection with current sample sizes and sampling locations.

Acknowledgments

We appreciate the collaboration of the Green Turtle Butchers Cooperative of Puerto Cabezas, Nicaragua, and we especially thank C. Clark and A. Salciano for assistance in sample collection. The study and manuscript were improved by J. Mortimer, C. Campbell, and R. Klinger. All samples were collected under permit by the Ministerio del Ambiente y Los Recursos Naturales (MARENA), Nicaragua, and exported to the United States under permits issued by Nicaraguan and US CITES authorities. Funding for field studies was provided by the Wildlife Conservation Society, Inter-American Foundation, the Chelonian Institute, the Nature Conservancy, and Sigma Xi to CJL. Genetic analyses were funded by National Science Foundation grant DEB-9225082 (Conservation and Restoration Biology Program) to BWB and a National Marine Fisheries Service contract to BWB and ALB. Protocols for the use of animals were approved by the University of Florida Animal Use and Care Committee.

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.

Bolten, A. B., K. A. Bjorndal, H. R. Martins, T. Dellinger, M. J. Biscoito, S. E. Encalada, and B. W. Bowen. 1998. Transatlantic developmental migrations of loggerhead sea turtles demonstrated by mtDNA sequence analysis. Ecol. Appl. 8:1–7.

Bowen, B. W., A. L. Bass, A. Garcia-Rodriguez, C. E. Diez, R. van Dam, A. Bolten, K. A. Bjorndal, M. M. Miyamoto, and R. J. Ferl. 1996. Origin of hawksbills turtles in a Caribbean feeding area as indicated by genetic markers. *Ibid.* 6:566–572.

Broderick , D., C. Moritz, J. D. Miller, M. Guinea, R. J. Prince, and C. J. Limpus. 1994. Genetic studies of the hawksbill turtle *Eretmochelys imbricata*: evidence for multiple stocks in Australian waters. Pac. Conserv. Biol. 1:123–131.

Carr, A. F. 1967. The sea turtle: so excellent a fishe. Natural History Press, Doubleday, Austin, TX.

———, and D. Goodman. 1970. Ecologic implications of size and growth in *Chelonia*. Copeia 1970 4: 783–786.

———, M. H. Carr, and A. B. Meylan. 1978. The ecology and migrations of sea turtles. No. 7. The West Caribbean green turtle colony. Bull. Am. Mus. Nat. Hist. 162:1–46.

Chapman, R. W. 1996. A mixed stock analysis of the green turtle: the need for null hypotheses, p. 137–146. *In:* Proceedings of the international symposium on sea turtle conservation genetics. B. W. Bowen and W. N. Witzell (eds.). NOAA Technical Memorandum NMFS-SEFSC-396. Miami, FL.

Encalada, S. E., P. N. Lahanas, K. A. Bjorndal, A. B. Bolten, M. M. Miyamoto, and B. W. Bowen. 1996. Phylogeography and population structure of the Atlantic and Mediterranean green turtle *Chelonia mydas*: a mitochondrial DNA control region sequence assessment. Mol. Ecol. 5:473–483.

FitzSimmons, N. N., C. J. Limpus, J. A. Norman, A. R. Goldizen, J. D. Miller, and C. Moritz. 1997. Philopatry of male marine turtles inferred from mitochondrial DNA markers. Proc. Nat. Acad. Sci. USA 94:8912–8917.

Hillis, D. M., B. K. Mable, A. Larson, S. K. Davis,

AND E. A. ZIMMER. 1996. Nucleic acids. IV. Sequencing and cloning, p. 321–381. *In:* Molecular systematics. D. M. Hillis, C. Moritz, and B. K. Mable (eds.). 2d ed. Sinauer Associates, Inc., Sunderland, MA.

INNIS, M. A., D. H. GELFAND, J. J. SNINSKY, AND T. J. WHITE. 1990. PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.

LAHANAS, P. N., K. A. BJORNDAL, A. B. BOLTEN, S. E. ENCALADA, M. M. MIYAMOTO, R. A. VALVERDE, AND B. W. BOWEN. 1998. Genetic composition of a green turtle feeding ground population: evidence for multiple origins. Mar. Biol. 130:345–352.

LIMPUS, C. J., J. D. MILLER, C. J. PARMENTER, D. REIMER, N. MCLACHLAN, AND R. WEBB. 1992. Migration of green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) turtles to and from Eastern Australian rookeries. Aust. Wildl. Res. 19:347–358.

MEYLAN, A. B. 1982. Sea turtle migration-evidence from tag returns, p. 91–100. *In:* Biology and conservation of sea turtles, proceedings of the world conference on sea turtle conservation. K. A. Bjorndal (ed.). Smithsonian Institution Press, Washington, DC.

MITCHELL, L. G., AND C. R. MERRILL. 1989. Affinity generation of single-stranded DNA for dideoxy sequencing following the polymerase chain reaction. Ann. Biochem. 178:239–242.

MORTIMER, J. A. 1981. The feeding ecology of the West Caribbean green turtle (*Chelonia mydas*) in Nicaragua. Biotropica 13:49–58.

NIETSCHMANN, B. 1972. Hunting and fishing focus among the Miskito Indians, Eastern Nicaragua. Hum. Ecol. 1:41–67.

OGREN, L. 1989. Status report of the green turtle, p. 89–94. *In:* Proceedings of the second western Atlantic turtle symposium. L. Ogren, F. Berry, K. Bjorndal, H. Kumpf, R. Mast, G. Medina, H. Reichart, and R. Witham (eds.). NOAA Tech. Memo. NMFS/SEFC-226. Miami, FL.

PARSONS, J. J. 1962. The green turtle and man. Univ. of Florida Press, Gainesville.

PELLA, J. J., AND G. B. MILNER. 1987. Use of genetic marks in stock composition analysis, p. 247–276 *In:* Population genetics and fisheries management. N. Ryman and F. Utter (eds.). Univ. of Washington Press, Seattle.

RICE, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223–225.

ROFF, D. A., AND P. BENTZEN. 1989. The statistical analysis of mitochondrial DNA polymorphisms: chi-square and the problem of small samples. Mol. Biol. Evol. 6:539–545.

ROSS, J. P., AND C. J. LAGUEUX. 1993. Tag return from a male green turtle. Mar. Turt. News. 62:5–6.

SOKAL, R. K., AND F. J. ROHLF. 1981. Biometry. 2d ed. W. H. Freeman and Co., San Francisco, CA.

SOLE, G. 1994. Migration of the *Chelonia mydas* population from Aves Island, p. 283–286. *In:* Proceedings of the fourteenth annual symposium on sea turtle biology and conservation. K. A. Bjorndal, A. B. Bolten, D. A. Johnson, and P. J. Eliazar (eds.). NOAA Tech. Memo. NMFS-SEFSC-351. Miami, FL.

ZAYKIN, D. V., AND A. I. PUDOVKIN. 1993. Two programs to estimate significance of χ^2 values using pseudo-probability tests. J. Hered. 84:152.

(AB) DEPARTMENT OF FISHERIES AND AQUATIC SCIENCES, UNIVERSITY OF FLORIDA, 7922 NW 71ST STREET, GAINESVILLE, FLORIDA 32653; (CL) DEPARTMENT OF WILDLIFE ECOLOGY AND CONSERVATION, UNIVERSITY OF FLORIDA, GAINESVILLE, FLORIDA 32611; AND (BB) DEPARTMENT OF FISHERIES AND AQUATIC SCIENCES, UNIVERSITY OF FLORIDA, 7922 NW 71ST STREET, GAINESVILLE, FLORIDA 32653; AND ARCHIE CARR CENTER FOR SEA TURTLE RESEARCH, 223 BARTRAM HALL, UNIVERSITY OF FLORIDA, GAINESVILLE, FLORIDA 32611. E-mail: abass@gnv.ifas.ufl.edu. Send reprint requests to AB. Submitted: 17 Nov. 1997. Accepted: 4 March 1998. Section editor: J. R. Gold.

APPENDIX. MITOCHONDRIAL DNA SEQUENCES OF THE 5′ END OF THE CONTROL REGION IN THE GREEN TURTLE, *Chelonia mydas*. The reference sequence is haplotype CM III (or A^{TAC}, accession number M98394, Allard et al., 1994). Polymorphic sites which define haplotype CM V are indicated by the base pair below the reference sequence. The first 71 base pairs in lowercase are the adjacent $tRNA^{PRO}$ with the actual control region sequence indicated by uppercase letters. Dashes indicate the position of the primers LTCM1 and HDCM1.

```
                                                                      60
tcaaaagaga aggacttaaa ccttcatccc cggtccccaa aaccggaatc ctataattaa
                                      ----- ---------- ----(LTCMI)
                                                                     120
actatccttt gACACAGGAA TAAAAGTGTC CACACAAACT AACTACCTAA ATTCTCTGCC
                                                                     180
GTGCCCAACA GAACAATACC CGCAATACCT ATCTATGTAT TATTGTACAT CTACTTATTT
                                                C  G
                                                                     240
ACCAATAGCA TATGACCAGT AATGTTAACA GTTGATTTGG CCCTAAACAT AAAAAATCAT
                          A
                                                                     300
TGAATTTACA TAAATATTTT AACAACATGA ATATTAAGCA GAGGATTAAA AGTGAAATGA
                                                                     360
CATAGGACAT AAAATTAAAC TATTATACTC AACCATGAAT ATCGTCACAG TAATTGGTTA
                       C                                      G
                                                                     420
TTTCCTAAAT AGCTATTCAC GAGAAATAAG CAACCCTTGT TAGTAAGATA CAACATTACC
                                                                     480
AGTTTCAAGC CCATTCAGTC TGTGGCGTAC ATAATTTGAT CTATTCTGGC CTCTGGTTAG
                   A
                                                                     540
TTTTTCAGGC ACATACAAGT AACGACGTTC ATTCGTTCCC CTTTAAAAGG CCTTTGGTTG
C                       G A
                                                                     600
AATGAGTTCT ATACATTAAA TTTATAACCT GGCATACGGT AGTTTTACTT GCATATAGTA
                   G       ------ ---------- ---(HDCM1)
                                                                     620
GTTTTTTTTC TCTCTGTGTT
```