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Population Structure and Diversity of Brazilian Green Turtle Rookeries Based on Mitochondrial DNA Sequences

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ABSTRACT. – **Mitochondrial DNA (mtDNA) sequences were determined for 168 green turtles (*Chelonia mydas*) nesting at the 3 known rookeries in Brazil: Trindade ($n = 99$), Atol das Rocas ($n = 37$), and Fernando de Noronha ($n = 16$). In addition, 32 green turtles were sampled on foraging grounds at Atol das Rocas and Fernando de Noronha. Significant genetic structure exists among the 3 rookeries, with haplotype frequencies significantly different between Trindade and the other 2 rookeries, and no significant difference between Atol das Rocas and Fernando de Noronha. In contrast to previous reports, we found no significant relationship between population size and mtDNA diversity when 14 Atlantic green turtle rookeries were compared for 3 measures of diversity. There was also no significant relation between rookery latitude and mtDNA diversity. Our results are consistent with the earlier hypothesis that haplotype CM-A8 is the closest relative to an ancestral Atlantic haplotype and the observation that CM-A8 is the most common and widespread haplotype in equatorial rookeries. Bayesian and hierarchical mixed stock models yielded similar estimates of rookery contributions to the Atol das Rocas and Fernando de Noronha foraging aggregation, with Ascension Island the primary source and with probable contributions from the Greater Caribbean and West Africa. This study provides data from the southwest Atlantic that are critical for an Atlantic-wide analysis of green turtle population structure.**

KEY WORDS. – **Reptilia; Testudines; Cheloniidae; *Chelonia mydas*; sea turtle; genetic diversity; population structure; Brazil**

Genetic tags, in the form of mtDNA sequences, have been used effectively in studies of the ecology and evolution of sea turtles (e.g., Norman et al. 1994; Bowen and Karl 1997; Bowen 2003). Rookeries (= nesting aggregations), including the Atlantic rookeries of green turtles (Encalada et al. 1996), can be distinguished by differences in their frequencies of mtDNA sequence variants. This population structure is attributed to natal homing in female turtles that limits gene flow among rookeries (Bowen 1995). mtDNA is a maternally inherited marker and therefore ideal for determining population structure in philopatric species, such as sea turtles.

The ability to distinguish among rookeries based on mtDNA haplotypes has allowed studies to address previously intractable questions, such as dispersal patterns of hatchlings away from nesting beaches and movement patterns of juveniles among developmental habitats. These studies require adequate sampling of sea turtle nesting aggregations. Although progress has been made in characterizing green turtle nesting and foraging aggregations in the Atlantic system, research is hampered by poor sampling in some regions, including the southwest Atlantic. For example, several mtDNA haplotypes identified in individuals on foraging grounds have not been found in rookery samples (Lahanas et al. 1998; Bass and Witzell 2000; Luke et al. 2004), which precludes identification of the source nesting population(s). In addition, recent advances in mixed stock analyses mandate much larger sample sizes to obtain reasonable confidence intervals around the estimated contributions to foraging aggregations (Bolker et al. 2003; Okuyama and Bolker 2005).

Our study identified mtDNA sequences in green turtles nesting at the 3 rookeries known to occur in Brazil and at 2 foraging grounds. Trindade is the site of the largest green turtle rookery in Brazil and the third largest in the Atlantic system (Moreira et al. 1995; Seminoff 2002), Atol das Rocas hosts the second largest green turtle rookery in Brazil (Bellini et al. 1995), and Fernando de Noronha Archipelago has a small green turtle rookery (Bellini and Sanches 1996). Providing data for these rookeries, particularly for Trindade, makes a substantial contribution towards satisfying one of the assumptions of mixed stock analyses—that all source rookeries are

sampled. Small foraging aggregations of juvenile green turtles also inhabit the waters around Atol das Rocas and Fernando de Noronha (Sanches and Bellini 1999). The only mtDNA sequence data previously available for these aggregations was a small sample ($n = 16$) of nesting turtles from Atol das Rocas (Encalada et al. 1996).

In this study, our objectives were to 1) increase our knowledge of mtDNA sequences in Atlantic green turtles; 2) evaluate degree of genetic differentiation among rookeries in Brazil; 3) assess the previously reported (Lahanas et al. 1994; Encalada et al. 1996) significant inverse relationship between rookery size and mtDNA diversity based on a larger number of rookeries and greater range of population sizes than those available for previous analyses; 4) determine whether the data from 3 Brazilian rookeries are consistent with the phylogeographic hypotheses presented by Encalada et al. (1996) for Atlantic green turtles; and 5) estimate rookery contributions to the foraging aggregations at Atol das Rocas and Fernando de Noronha using 2 methods recently developed for mixed stock analyses.

METHODS

Study Sites, Sample Collection, and mtDNA Sequence Analysis. — The locations of the 3 study sites are shown in Fig. 1. Trindade (20°30′S, 29°49′W) has a total area of 8.2 km^2 and lies 1200 km off the southeast coast of Brazil (Moreira et al. 1995). Atol das Rocas (3°52′S, 33°49′W) has a total area of 7.2 km^2 and lies 270 km off the northeast coast of Brazil (Bellini et al. 1995). Fernando de Noronha Archipelago (3°50′S, 32°24′W) has an area of 26 km^2 and lies 380 km off the northeast coast of Brazil (Bellini and Sanches 1996).

Skin samples were collected with sterile 6-mm biopsy punches from the neck area of female green turtles when they came ashore to nest at Trindade in the 1998–1999 nesting season, Atol das Rocas in 2000, and Fernando de Noronha in the 1998–1999 and 1999–2000 nesting seasons. Tissue samples were collected in the same way from green turtles captured by divers at the foraging grounds of Atol das Rocas in 2000 and Fernando de Noronha in 1999. Samples were preserved in a saturated NaCl aqueous solution (250 mM EDTA, pH 7.5; 20% DMSO) and stored at room temperature. To avoid resampling individual turtles, an Inconel alloy tag with an identification number was applied to each front flipper of all sampled turtles before their release.

DNA extractions were conducted at the Genetics Analysis Lab at the University of Florida with standard phenol/chloroform methodology (Hillis et al. 1996). A 481 base-pair (bp) fragment at the 5′ end of the control region of the mitochondrial genome was amplified via polymerase chain reaction (PCR; Mullis and Faloona 1987) using primers LTCM2 and HDCM2 (Encalada et al. 1996). The PCR amplifications included one cycle at 94°C (1 min) followed by 35 cycles at 94°C (45 s), 55°C (30 s), and 72°C (45 s) and a final 3-min extension at 72°C. Standard precautions, including negative controls (template-free PCR reactions), were used to test for contamination and to assure the reliability of PCR reactions (Innis et al. 1990).

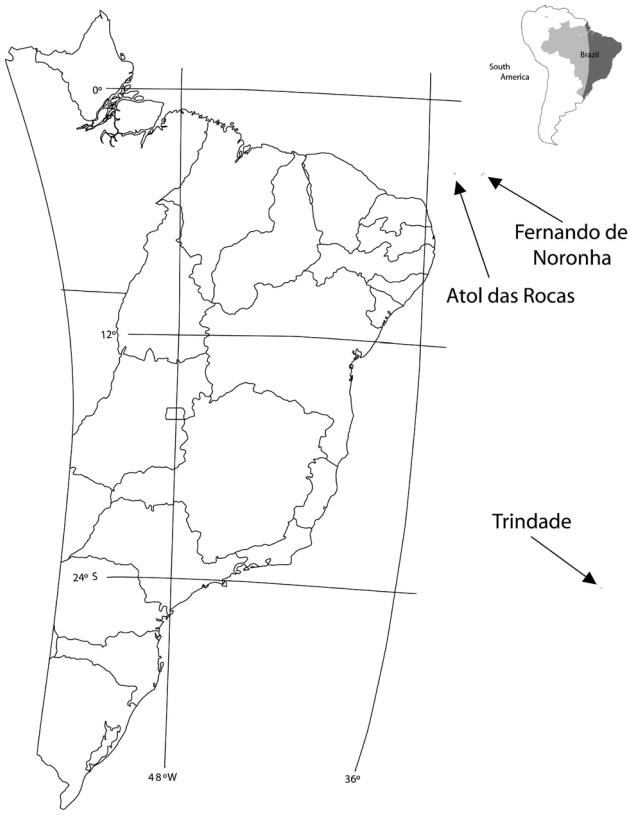

Figure 1. Map of the east coast of Brazil; arrows indicate the locations of the 3 study sites.

Cycle sequencing reactions with fluorescently labeled dideoxynucleotides were performed and sequencing products were analyzed with an automated DNA sequencer (Applied Biosystems model 373A) at the DNA Sequencing Core at the University of Florida. Sequences were aligned using the program Sequencher (v.3.1.1, Gene Codes Corporation), compared to known haplotypes, and collated for analysis. All samples were sequenced in the forward direction; novel haplotypes were also sequenced in the reverse direction to assure the accuracy of DNA sequence designations. Haplotype designations were assigned according to the Marine Turtle DNA sequences website maintained by the Archie Carr Center for Sea Turtle Research at the University of Florida (http://accstr.ufl.edu/genetics.html).

Data Analyses. — For analyses, the sample of nesting females from Atol das Rocas was combined with a previously published sample collected prior to 1991 ($n = 16$, Encalada et al. 1996). The probability that an individual female was sampled in both sampling periods (before 1991 and in 2000) is quite low because all turtles were tagged, mortality of turtles sampled in the first period would be substantial between the 2 sampling periods, and

the proportion of sampled turtles is small. Therefore, we believe the "combined" sample ($n = 53$) is a valid sample. All analyses of haplotype diversity (h), nucleotide diversity (π), and analysis of molecular variance (AMOVA) were conducted using the software Arlequin (ver. 2.000; Schneider et al. 2000). All analyses that involved estimates of sequence divergence used the Tamura–Nei model of nucleotide substitutions (Tamura and Nei 1993) following Bowen et al. (2004, 2005).

Spatial population structuring was evaluated with AMOVA by partitioning variance within and among the 3 nesting aggregations. Significance was assessed by comparison to values generated from at least 20,000 random permutations of haplotypes among the aggregations. Haplotype frequencies among nesting aggregations and between foraging ground aggregations were compared with chi-square tests using the computer program CHIRXC (Zaykin and Pudovkin 1993), which calculates probabilities of independence using a Monte Carlo randomization method (1000 iterations). Two mixed stock analyses were employed: the Bayesian method described in Bolker et al. (2003) and the hierarchical method that includes rookery population size as a covariate (Okuyama and Bolker 2005). Spearman Rank correlations were conducted with S-PLUS software (version 6.1, 2002; Insightful Corporation, Seattle, WA).

RESULTS AND DISCUSSION

mtDNA Sequences, and Rookery Structure and Diversity. — At the 3 rookeries, 168 female green turtles were sampled as they came ashore to nest, and 32 green turtles were sampled on foraging grounds. Green turtles sampled on foraging grounds at Atol das Rocas ranged in size from 37 to 108 cm curved carapace length (mean = 62.1 cm, $n = 23$); those on Fernando de Noronha foraging grounds ranged from 38 to 74.5 cm curved carapace length (mean = 50.7 cm; $n = 9$). Thirteen haplotypes were found in the 200 turtles (Table 1). Overlap of haplotypes between these rookeries and foraging grounds was not complete; 7 rookery haplotypes were not reported from the foraging grounds, and 3 foraging ground haplotypes were not found in the rookery samples. We found one previously unreported haplotype (CM-A33). The sequence of haplotype CM-A33 (GenBank #AF366262; posted at http://accstr.ufl.edu/genetics.html) aligns most closely with CM-A32, from which it differs by a transition (A to G) at bp 349, based on the presence of adenine at this position in all other known green turtle haplotypes. Our rookery sample from Atol das Rocas added 3 haplotypes (CM-A10, CM-A25, CM-A32) from the previously published sample ($n = 16$; Encalada et al. 1996).

Genetic structuring among the 3 Brazilian rookeries was significant, but relatively low (AMOVA, $\Phi_{st} = 0.0251$, $p = 0.039$). Pairwise comparisons of the 3 rookeries indicated that the haplotype frequencies of Atol das Rocas and Fernando de Noronha, the 2 smaller rookeries separated by approximately 155 km, are not significantly different from each other, whereas both rookeries are significantly different from the larger and more distant (ca.1850 km) Trindade rookery (chi-square tests, Table 2).

Three measures of mtDNA diversity—number of haplotypes, haplotype diversity, and nucleotide diversity—were calculated using the same methods for the 3 Brazil rookeries and for 11 other rookeries from data presented in the references cited in Table 3. Rookeries at Trindade and Atol das Rocas had similar diversity indices (Table 3). Compared to the other Atlantic rookeries, the rookeries at Trindade and Atol das Rocas are characterized by relatively high diversity as measured by number of haplotypes and haplotype diversity. However, their nucleotide diversities are relatively low because of the low sequence divergences among haplotypes. All 3

Table 1. Numbers of each mtDNA haplotype present in samples of adult female green turtles at 3 rookeries and green turtles at 2 foraging grounds in Brazil (n = sample size).

	Rookeries			Foraging grounds	
Haplotype	Trindade ($n = 99$)	Atol das Rocas ($n = 53$)	Fernando de Noronha ($n = 16$)	Atol das Rocas ($n = 23$)	Fernando de Noronha ($n = 9$)
CM-A5				5	
CM-A6				2	
CM-A8	67	36	14	13	7
CM-A9	19	7		2	1
CM-A10		2			1
CM-A11	1	1			
CM-A12		5			
CM-A23	6				
CM-A24	1				
CM-A25		1	2		
CM-A32	4	1			
CM-A33	1				
CM-A46				1	

Table 2. Comparisons of haplotype frequencies among the 3 Brazil rookeries. Chi-square values (p value); significant values are in bold.

	Trindade	Atol das Rocas
Atol das Rocas	**20.638 (0.005)**	
Fernando de Noronha	**18.315 (0.027)**	8.664 (0.180)

measures of diversity for the Fernando de Noronha rookery are at the lower ends of the continua of Atlantic rookeries. Nucleotide diversity for Fernando de Noronha is 0 because the 2 haplotypes recorded from that population only differ by a 10-bp indel. The extent to which the low diversity in the Fernando de Noronha rookery is an artifact of sample size ($n = 16$) is not known. When all Atlantic rookeries are analyzed, none of the 3 diversity indices was significantly related to sample size (Spearman Rank correlations, $0.128 \leq p \leq 0.583$). However, a sample of 16 turtles may well be insufficient to capture the diversity of the nesting aggregation.

Significant inverse relationships between population size and both haplotype diversity and nucleotide diversity have been reported for Atlantic green turtle rookeries (Lahanas et al. 1994; Encalada et al. 1996). In recent years, more Atlantic green turtle rookeries have been sampled, and sample sizes from previously sampled rookeries have been increased. Analyses based on the larger number of rookeries now available reveal no significant relationship between population size and any of the 3 measures of diversity (Spearman Rank correlations, $0.127 \leq p \leq 0.414$, Fig. 2a).

mtDNA Sequences on Foraging Grounds and Mixed Stock Analyses. — Haplotype frequencies of the foraging ground aggregations at Atol das Rocas and Fernando de Noronha were not significantly different (chi-square = 6.194, $p = 0.327$), so they were combined for mixed stock analyses. From both mixed stock analyses, we can conclude that the largest contribution to the combined foraging ground of Atol das Rocas and Fernando de Noronha is from Ascension Island (point estimates of 0.69 or 0.53; Fig. 3) and that the foraging aggregation represents a mixed stock with probable contributions from the Greater Caribbean and West Africa. The relative contributions from rookeries should be evaluated with caution because many of the 95% confidence intervals are broad and all, except Ascension, encompass 0. Incorporating rookery size in the mixed stock analysis does not have substantial effects on the proposed rookery contributions; point estimates decrease for Ascension and Aves, whereas point estimates increase for Costa Rica and Trindade. Based on current knowledge of rookery genetic composition, the individuals with haplotype CM-A5 on the Brazilian foraging grounds are probably derived from the Greater Caribbean, although the São Tomé rookery is also a possible source. Of 20 green turtles sampled at the São Tomé rookery, one had a CM-A5 haplotype (Formia 2002).

The results of the mixed stock analyses are supported by movement data documented from flipper tags. Female green turtles tagged at nesting beaches on Ascension Island and Surinam overlap in foraging areas off the east

Table 3. Number of mtDNA haplotypes, haplotype diversity (± SD), nucleotide diversity (± SD), and approximate number of nesting females per year for Atlantic green turtle rookeries (n = number of individuals sampled). Rookery abbreviations are used in Fig. 3.

	n	No. of haplotypes	Haplotype diversity	Nucleotide diversity	Number of females
Trindade (TR)	99[a]	7	0.5046 ± 0.0522	0.0012 ± 0.0011	3000[b]
Atol das Rocas (AR)	53[a]	7	0.5196 ± 0.0763	0.0012 ± 0.0011	115[c]
Fernando de Noronha (FN)	16[a]	2	0.2333 ± 0.1256	0	10[d]
Costa Rica (CR)	433[e]	5	0.1627 ± 0.0231	0.0033 ± 0.0022	24,000[b]
Mexico (MX)	20[f]	7	0.8158 ± 0.0575	0.0052 ± 0.0033	1547[b]
Florida (FL)	36[f,g]	4	0.6238 ± 0.0489	0.0051 ± 0.0031	759[b]
Aves (AV)	55[f,g]	2	0.1374 ± 0.0599	0.0029 ± 0.0020	267[b]
Surinam (SU)	46[f,g]	4	0.1671 ± 0.0733	0.0011 ± 0.0010	1800[b]
Ascension Island (AI)	207[f,h]	10	0.3058 ± 0.0417	0.0007 ± 0.0008	4000[h]
Guinea Bissau (GB)	70[f,h]	1	0	0	1500[h]
Bioko (BI)	50[h]	2	0.1837 ± 0.0681	0.0004 ± 0.0006	500[h]
São Tomé (ST)	20[h]	7	0.5842 ± 0.1270	0.0030 ± 0.0021	90[h]
Príncipe (PR)	6[h]	2	0.5333 ± 0.1721	0.0011 ± 0.0012	90[h]
Cyprus (CY)	9[f]	2	0.2222 ± 0.1662	0.0005 ± 0.0007	100[i]

[a] This study.
[b] Seminoff (2002).
[c] Bellini et al. (1995).
[d] Bellini and Sanches (1996).
[e] Bjorndal, Bolten, and Troëng (2005).
[f] Encalada et al. (1996).
[g] Bjorndal and Bolten, unpubl. data, 2005.
[h] Formia (2002).
[i] Broderick et al. (2002).

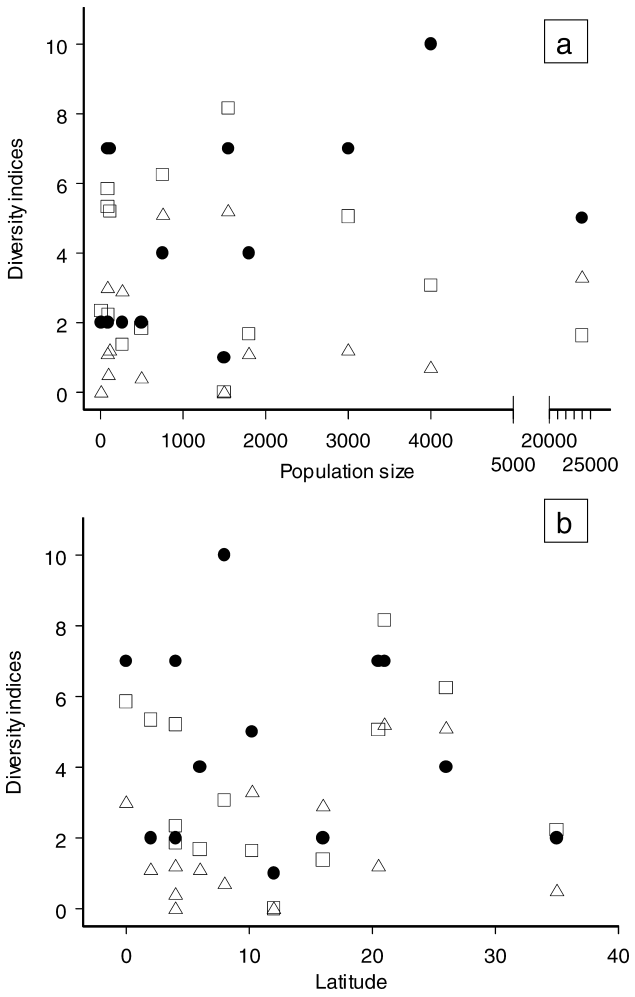

Figure 2. Relationship between 3 diversity indices (solid circles number of haplotypes, open boxes haplotype diversity × 10, open triangles nucleotide diversity × 1000) and (a) population size (= annual number of nesting females) and (b) latitude. No relationships are significant; analyzed on actual values.

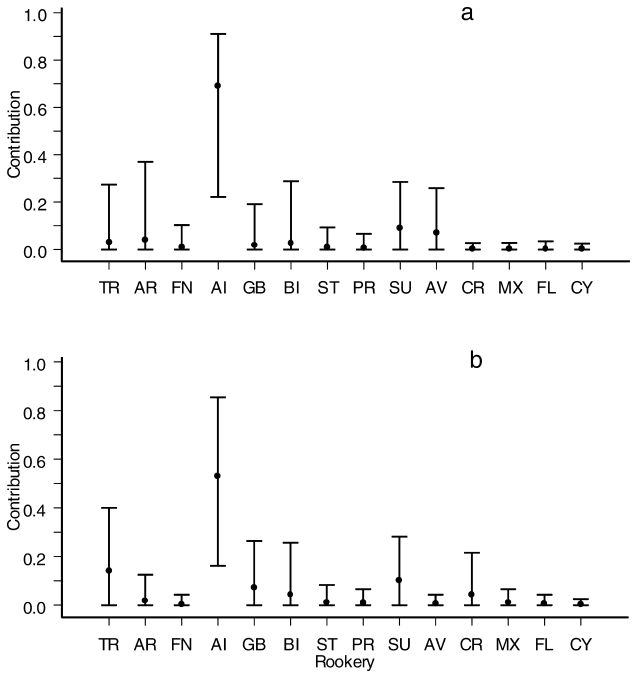

Figure 3. Proportional contributions of rookeries to the combined foraging aggregation of Atol das Rocas and Fernando de Noronha based on (a) the Bayesian method described in Bolker et al. (2003) and (b) the hierarchical method that includes population size as a covariate (Okuyama and Bolker 2005). Bars represent 95% confidence intervals. Rookery abbreviations are given in Table 3.

coast of Brazil (Carr 1975). Three subadult green turtles tagged off the northeast coast of Brazil have been recovered in the waters of the Caribbean—one in Trinidad (Lum et al. 1998) and two in Nicaragua (Lima et al. 1999, 2003). A green turtle tagged while nesting at Tortuguero, Costa Rica, was recovered in the waters of northeast Brazil (Lima and Troëng 2001), and a female tagged while nesting on Trindade was recaptured in Senegal (Marcovaldi et al. 2000). The feeding aggregations in Atol das Rocas and Fernando de Noronha are small relative to aggregations at Almofala and Ubatuba on the north and south coasts of Brazil, respectively. Mixed stock analyses of these foraging aggregations currently underway (E. Naro and M.Â. Marcovaldi, unpubl. data, 2005) should yield valuable information on the rookery sources of green turtles in Brazilian waters. Mixed stock analyses, flipper tagging, and satellite telemetry contribute to our understanding of the complex movements of green turtles within the Atlantic system, which is critical in setting boundaries for meta-population models now being developed (Bolten and Chaloupka 2004).

Implications for Phylogeography of Atlantic Green Turtles. — Our data from Brazil provide insights on previous phylogeographic observations and hypotheses. First, the haplotypes from the 3 Brazilian rookeries are consistent with the suggestion that haplotype CM-A8 may represent the closest relative to an ancestral Atlantic haplotype and the observation that CM-A8 is the most common and widespread haplotype in equatorial rookeries (Encalada et al. 1996). Our results and those of Formia (2002) indicate that CM-A8 is the most common haplotype in the 8 Atlantic rookeries southeast of Surinam that have been sampled to date and remains in the central position of a network of haplotypes (Formia 2002). Of the 168 females sampled at Brazilian rookeries, 117 (70%) are haplotype CM-A8.

Second, Encalada et al. (1996) hypothesized that equatorial regions served as refugia for green turtles during Pleistocene glacial maxima with colonizations into higher latitudes during interglacial periods. If this is true, genetic diversity within rookeries would be expected to decline with latitude or distance from the refugia. However, there is no significant relation between latitude and any of the 3 indices of mtDNA diversity (Spearman Rank Correlations, $0.354 \leq p \leq 0.889$, Fig. 2b), and the 2 rookeries with the highest haplotype and nucleotide diversities (Mexico and Florida) are among the most distant rookeries. Although it

may appear in Fig. 2b that the lack of a significant relationship is caused by Cyprus, at the highest latitude, excluding Cyprus from the analyses did not change the lack of significance (Spearman Rank correlations, $0.131 \leq p \leq 0.992$). These findings are not incompatible with the equatorial refugia hypothesis, but they do suggest that the process of rookery establishment in Atlantic green turtles has been more complex than a simple outward movement from a refugium. Data on nuclear DNA would enhance the evaluation of patterns of colonization in sea turtle rookeries.

Third, Bjorndal et al. (2005) noted that the occurrence of the two most common haplotypes in green turtles in the Greater Caribbean, CM-A3 and CM-A5, are inversely related in foraging aggregations. That is, the ratio of CM-A3 to CM-A5 declines from west to east. The presence of CM-A5 and lack of CM-A3 in the combined Atol das Rocas and Fernando de Noronha foraging sample continues that trend along the west–east axis.

In conclusion, our results will make a substantial contribution to studies of genetic structure of green turtle rookeries throughout the Atlantic and to mixed stock analyses of green turtle foraging aggregations in the Atlantic. Research efforts should be directed to sampling previously unsampled rookeries and foraging aggregations and increasing sample sizes in those that have been sampled. Evaluations of genetic structure and patterns of genetic diversity would be improved by including other genetic markers.

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