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Population structure and genetic diversity in green turtles nesting at Tortuguero, Costa Rica, based on mitochondrial DNA control region sequences

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Abstract The green turtle (*Chelonia mydas*) nesting population at Tortuguero, Costa Rica, is the largest nesting aggregation in the Atlantic, by at least an order of magnitude. Previous mitochondrial DNA (mtDNA) surveys based on limited sampling ($n = 41$) indicated low genetic diversity and low gene flow with other Caribbean nesting colonies. Furthermore, a survey of nuclear DNA diversity invoked the possibility of substructure within the Tortuguero rookery. To evaluate these characteristics, mtDNA control region sequences were determined for green turtles nesting at Tortuguero in 2001 ($n = 157$) and 2002 ($n = 235$). The increased sample revealed three additional haplotypes; five haplotypes are now known for Tortuguero female green turtles. Analyses of molecular variance indicated that there was no significant spatial population structure along the 30-km nesting beach. In addition, no temporal population structure was detected either between the two nesting seasons or within the nesting season. As a result of the larger sample size and additional haplotypes, estimates of genetic separation among Caribbean nesting colonies have changed and the concordance of phylogenetic and phylogeographic patterns reported in the past for green turtles in the Greater Caribbean has weakened. The five haplotypes from Tortuguero represent 36% of the haplotypes identified in green turtle nesting aggregations in the Greater Caribbean and 17% of the haplotypes known to occur in nesting or foraging aggregations in the Greater Caribbean. Haplotype

diversity (0.16) and nucleotide diversity (0.0034) for the Tortuguero population are substantially lower than those for the combined rookeries in the Greater Caribbean (0.44 and 0.0078, respectively). Although comprehensive evaluation of regional genetic diversity requires nuclear DNA data, our study indicates that conserving genetic diversity in Caribbean green turtles will require careful management of the smaller rookeries in addition to the Tortuguero rookery.

Introduction

Mitochondrial DNA (mtDNA) sequences have been used increasingly in studies of the ecology and evolution of sea turtles (Norman et al. 1994; Bowen and Karl 1997; Bowen 2003). Differences in the frequency of mtDNA sequences can distinguish among rookeries (nesting aggregations) including the Atlantic rookeries of green turtles, *Chelonia mydas* (Encalada et al. 1996). This population structure is attributed to natal homing in female turtles that limits gene flow among rookeries. Studies based on genetic tags and flipper tags have revealed that females return to their natal beach to nest and exhibit strong site-fidelity to that beach or region throughout their reproductive lives (Carr et al. 1978; Bowen 1995). Although “mistakes” in natal homing must have occurred for new rookeries to be established, genetic evidence indicates that such mistakes are rare between geographically distinct rookeries.

The ability to distinguish among rookeries based on mtDNA haplotype frequencies have allowed studies to address previously intractable questions. Most sea turtle species exhibit complex life history patterns (Bolten 2003) that involve an early dispersal of hatchlings from the nesting beaches into oceanic waters. After several years, unknown cues prompt immature turtles to shift to neritic foraging grounds where they may undertake extensive developmental migrations among neritic foraging grounds until sexual maturity is attained, usually after decades. Once mature, sea turtles make periodic

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reproductive migrations to nesting beaches that may be thousands of kilometers from their foraging areas. Migratory patterns of sea turtles across ocean basins have been clarified using mtDNA haplotypes (Bowen et al. 1995; Bolten et al. 1998). Rookery sources have been identified for mixed stocks of sea turtles on foraging grounds (Broderick et al. 1994; Lahanas et al. 1998; Luke et al. 2004) and for turtles killed by direct take by humans or indirect take in commercial fisheries (Bowen et al. 1995; Bass et al. 1998; Laurent et al. 1998).

These studies, particularly those assigning rookery sources to turtles on their foraging grounds, require adequate sampling of sea turtle nesting aggregations. Several haplotypes identified in individuals on foraging grounds have not been found in rookery samples (e.g., Bolten et al. 1998; Lahanas et al. 1998), and this precludes accurate identification of source nesting populations. 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). The nesting beach at Tortuguero (N10°35.51 W83°31.40 to N10°21.46 W83°23.41) on the northeast coast of Costa Rica supports the largest nesting colony of green turtles in the Atlantic, by at least an order of magnitude (Seminoff 2002). In recent years, approximately 20,000–40,000 female green turtles have nested at Tortuguero each year (Bjorndal et al. 1999; Solow et al. 2002; Troëng and Rankin 2005). However, the previous genetic sample from Tortuguero consisted of only 41 female green turtles (Lahanas et al. 1998).

Genetic tags can also be used to evaluate spatial and temporal structuring within a nesting population. The Tortuguero nesting beach is on an island, separated from the mainland by the Tortuguero River. Nesting activity has consistent spatial and temporal patterns (Tiwari et al., in press). Spatial distribution has a strong central tendency and diminishes to north and south along the 30-km nesting beach. Each year, almost all green turtles nest between mid June and early November, but a few deposit nests in every month of the year (Carr et al. 1978). The number of nesting turtles varies greatly among years, often in alternating high and low years (Bjorndal et al. 1999; Solow et al. 2002; Troëng and Rankin 2005). The extent to which these spatial and temporal patterns are a result of genetic structuring within the population is not known. However, a survey with minisatellite DNA suggested that relatedness of nesting females decreased with increasing distance along the northernmost 8 km of the rookery (Peare and Parker 1996). These findings indicate that natal homing may be sufficiently precise to induce population genetic structure within the 30-km nesting beach.

To evaluate temporal and spatial structure, we determined mtDNA haplotypes for 392 female green turtles nesting at Tortuguero in 2001 ($n = 157$) and 2002 ($n = 235$). The population was sampled to allow evaluation of temporal population structuring between

nesting cohorts in 2001 and 2002 and within the 2002 nesting season and assessment of spatial population structuring in both years along the entire 30-km nesting beach. In addition, we compare haplotype and nucleotide diversity for the large Tortuguero rookery with those of small rookeries in the Greater Caribbean region. We also evaluate genetic separation and gene flow among Caribbean rookeries, the proposed phylogeographic division between green turtle rookeries in the eastern and western Caribbean (Lahanas et al. 1994; Encalada et al. 1996), and past mixed stock analyses (Bass et al. 1998; Lahanas et al. 1998; Bass and Witzell 2000; Luke et al. 2004) in light of the larger sample from Tortuguero.

Materials and methods

Sample collection and mtDNA sequence analysis

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 Tortuguero, Costa Rica. Samples were preserved in a saturated NaCl aqueous solution with EDTA and DMSO (FitzSimmons et al. 1999) or 70% ethanol and stored at room temperature. To avoid re-sampling individual turtles, an Inconel alloy tag with an identification number was applied to each front flipper of all turtles sampled, except 5 females that had been killed by jaguars. All samples were collected by the team of staff and volunteers of the Caribbean Conservation Corporation that monitors the nesting sea turtles at Tortuguero each year.

To evaluate spatial structuring along the 30-km beach, samples were collected in northern, central and southern sections in both years. From north to south, the three 7.2-km spatial sections were between 0 and 7.2 km, 12.1 and 19.3 km, and 21.7–29.0 km (using the traditional mile markers at Tortuguero, these sections are bounded by the 0–4.5, 7.5–12, and 13.5–18 mile markers, respectively). Five turtles sampled in the areas between the three sections were not included in the spatial analysis. To evaluate temporal structuring within the nesting season, samples in 2002 were collected from females nesting in three periods: before the start of the main nesting season (18 March–31 May), during the first half of the season (28 July–6 September), and during the second half of the season (11 September–22 October). Only data for 2002 were used for temporal sub-groups because samples were not collected over the temporal range in 2001. In 2001, seven samples were collected before the main season, 150 in the first half of the season, and none in the second half of the season.

DNA isolations 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 fragment at the 5' end of the control region of the mitochondrial genome was amplified via polymerase chain reaction (PCR) methodology (Mullis

and Falooa 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 fidelity of PCR reactions (Innis et al. 1990).

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 Clustal X v1.81 (Thompson et al. 1997). 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

Haplotypes not previously reported from green turtle rookeries were fitted to an unrooted parsimony network presented by Encalada et al. (1996) and modified as described below. This unrooted parsimony network was confirmed using the program TCS v1.18 (Clement et al. 2000). All analyses of haplotype diversity (h), nucleotide diversity (π), genetic distance based on haplotype frequencies (F_{st}), gene flow (N_m based on F_{st} estimates, equivalent to the effective number of migrants between rookeries per generation), 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 with no gamma correction (Tamura and Nei 1993).

Spatial and temporal population structuring was evaluated with AMOVA by partitioning variance within

and among population spatial and temporal sub-groups. We assessed the proportion of genetic variance among either temporal or spatial population sub-groups with haplotype frequencies alone and incorporating sequence divergences among haplotypes (Φ_{st}). In all cases, the two approaches yielded the same conclusions, so only Φ_{st} values based on the latter analyses are reported here. Significance was assessed by comparison to values generated from at least 20,000 random permutations of haplotypes among population sub-groups.

A chi-square test using the computer program CHIRXC (Zaykin and Pudovkin 1993), which calculates probabilities of independence using a Monte Carlo randomization method (1000 iterations), was used to test for differences between the haplotype frequency of the Tortuguero nesting population and previously published frequencies of green turtle aggregations on foraging grounds in Nicaragua, southern Bahamas, Florida, and Barbados.

For comparative purposes, a sample for rookeries in the Greater Caribbean (13 haplotypes, $n=590$) was compiled from five rookeries: the Combined Tortuguero sample ($n=433$), Mexico ($n=20$), Florida ($n=36$), Aves Island ($n=55$) and Suriname ($n=46$). Data for these five rookeries are from Encalada et al. (1996), Lahanas et al. (1998), and unpublished data (Bjorndal and Bolten).

Results

Our sample increases the number of known haplotypes in Tortuguero female green turtles from two to five. In both 2001 and 2002, four mtDNA haplotypes were present in the samples: haplotypes CM-A3, CM-A5, CM-A20, and CM-A21 (Table 1). Haplotype CM-A5 had not previously been reported from Tortuguero, but had been recorded at other rookeries (Lahanas et al. 1998). Haplotypes CM-A20 and CM-A21 had previously only been reported from a foraging ground in the southern Bahamas (Lahanas et al. 1998) and were not

Table 1 Haplotype frequencies, haplotype diversities ($h \pm SD$), and nucleotide diversities ($\pi \pm SD$) for female green turtles (*Chelonia mydas*) nesting at Tortuguero, Costa Rica

	2001	2002	2001 + 2002	1988	1988 + 1996	Combined	Greater Caribbean
Sample size	157	235	392	15	41	433	590
Haplotype							
CM-A3	142	213	355	14	40	395	
CM-A4	0	0	0	1	1	1	
CM-A5	13	19	32	0	0	32	
CM-A20	1	1	2	0	0	2	
CM-A21	1	2	3	0	0	3	
Number of haplotypes	4	4	4	2	2	5	13
Haplotype diversity (h)	0.18 ± 0.04	0.17 ± 0.03	0.17 ± 0.02	0.13 ± 0.11	0.05 ± 0.05	0.16 ± 0.02	0.44 ± 0.02
Nucleotide diversity (π)	0.0037 ± 0.0024	0.0036 ± 0.0023	0.0037 ± 0.0024	0.0003 ± 0.0005	0.0001 ± 0.0003	0.0034 ± 0.0022	0.0078 ± 0.0044

Six samples are presented: 2001, 2002, 2001 + 2002, 1988 (Encalada et al. 1996), 1988 + 1996 (Lahanas et al. 1998), and Combined (1988 + 1996 + 2001 + 2002). Haplotype frequencies are not

significantly different among the six Tortuguero samples; haplotype frequencies are significantly different between the Combined Tortuguero sample and the Greater Caribbean sample (see text)

Table 2 Haplotype frequencies and Φ_{st} values from AMOVA analyses for three spatial sub-groups of the 2001/2002 sample (north, central and south) and three temporal sub-groups of the 2002 sample (pre-season, first half of season, second half of season) of the population of female green turtles (*Chelonia mydas*) nesting at Tortuguero, Costa Rica

	Spatial sub-groups			Temporal sub-groups		
	North	Central	South	Pre-season	First half	Second half
Sample size	130	119	138	30	107	98
Haplotype						
CM-A3	118	108	124	24	97	92
CM-A5	12	9	11	4	10	5
CM-A20	0	1	1	0	0	1
CM-A21	0	1	2	2	0	0
Φ_{st}	-0.0075 ($p=0.961$)			0.0201 ($p=0.091$)		

known from a rookery. Haplotype CM-A4, previously reported from Tortuguero, was not found in our samples in 2001 or 2002.

Haplotype frequencies, haplotype diversities, and nucleotide diversities for six Tortuguero samples (from four different years and combinations) are presented in Table 1. There are no significant differences among the haplotype frequencies (AMOVA, $\Phi_{st}=0.0010$, $p=0.308$) for the six Tortuguero samples. Differences between the Greater Caribbean sample and the Combined Tortuguero sample are significant (AMOVA, $\Phi_{st}=0.0772$, $p<0.0001$).

No significant temporal or spatial structuring within the Tortuguero nesting population was evident from mtDNA haplotypes (AMOVA, Table 2). However, because over 90% of the individuals have one haplotype, these conclusions should be tempered by a recognition

of the limited statistical power. Spatial structuring in the two annual sub-groups (2001 and 2002) was not significantly different ($\Phi_{st}=-0.0053$, $p=1$). Temporal structuring among the three temporal sub-groups approached significance (Table 2) because the only CM-A21 haplotypes ($n=2$) occurred in the pre-season sample and the only CM-A20 haplotype ($n=1$) occurred in the second half sample. The absence of these uncommon haplotypes from the first half of the season is a function of sampling and is not consistent among seasons; in 2001, females with haplotypes CM-A20 and CM-A21 were sampled during the first half of the season.

The unrooted parsimony network presented in Encalada et al. (1996) for the mtDNA haplotypes known from Atlantic green turtle rookeries was revised (Fig. 1) to include CM-A20 and CM-A21, now known to occur at Tortuguero. CM-A27, which has been reported from a nesting beach at Playa Antonio, on the southwest coast of Cuba (Espinosa López et al. 2000), is the haplotype previously identified as “Hypothetical 1” in Encalada et al. (1996). In addition, we deleted the “Hypothetical 2” haplotype proposed by Encalada et al. (1996) and attached CM-A18 to CM-A1, with a homoplasmy at site 167, as suggested by Brower (1999). These changes were confirmed using the program TCS v1.18. Female green turtle haplotypes at Tortuguero are now divided between Cluster A (CM-A3, CM-A4) and Cluster B (CM-A5, CM-A20, CM-A21). In the 2001/2002 sample, 90.6% and 9.4% are derived from Cluster A and B, respectively. In the combined sample, the values are 91.5% and 8.5%.

Genetic structuring (Φ_{st}) among the five Caribbean rookeries is significant with either Tortuguero sample (Table 3), but the point estimate decreased with the larger, combined Tortuguero sample. Pairwise comparisons revealed that the larger Tortuguero sample resulted in slightly greater estimates of genetic separation between Tortuguero and Mexico and Tortuguero and Florida, whereas estimates of genetic separation decreased slightly between Tortuguero and the eastern Caribbean rookeries of Aves and Surinam (Table 3). Because all N_m estimates are below 1 (Table 3), gene flow between Tortuguero and the other rookeries is apparently insufficient to maintain homogeneous genotype frequencies (Slatkin 1987).

Fig. 1 Parsimony network modified from Encalada et al. (1996) as described in the text and confirmed with the program TCS v1.18. Haplotypes from rookeries in the Greater Caribbean are in squares; dark squares are haplotypes found at Tortuguero; hexagons are haplotypes from rookeries outside of the Greater Caribbean (8–12 from the South Atlantic, 13 and 14 from the Mediterranean). Mutation site numbers are identified along branches, and asterisks indicate assumed homoplasmy

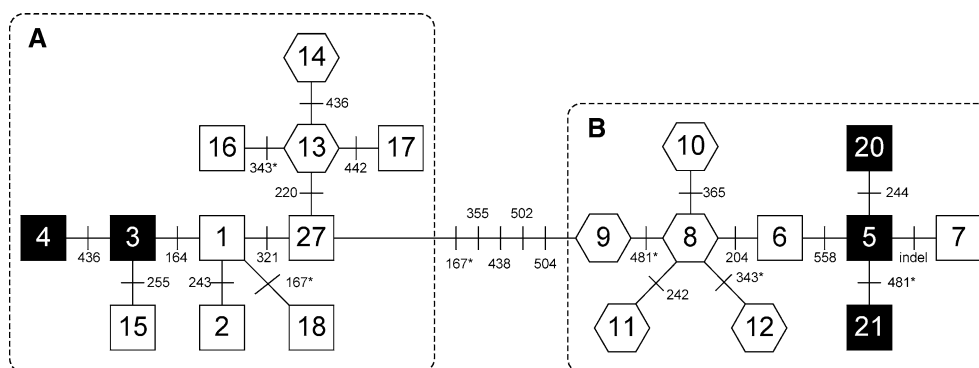


Table 3 Φ_{st} values (p value) from AMOVA analysis of the five Greater Caribbean rookeries, and F_{st} values (p value) and N_m values from pairwise comparisons between four rookeries (data from Lahanas et al. 1998) and two samples for Tortuguero: 1988+1996 ($n=41$) and Combined ($n=433$, see Table 1 for description of samples)

	Tortuguero 1988 + 1996 sample	Tortuguero combined sample
AMOVA Φ_{st}		
Five rookeries	0.83 (0.00)	0.71 (0.00)
Pairwise F_{st}		
Mexico ($n=20$)	0.53 (0.00)	0.61 (0.00)
Florida ($n=24$)	0.47 (0.00)	0.51 (0.00)
Aves ($n=30$)	0.88 (0.00)	0.80 (0.00)
Surinam ($n=15$)	0.89 (0.00)	0.81 (0.00)
Pairwise N_m		
Mexico ($n=20$)	0.44	0.32
Florida ($n=24$)	0.56	0.48
Aves ($n=30$)	0.07	0.12
Surinam ($n=15$)	0.06	0.11

Discussion

Population samples

A recurring concern in population genetics is whether or not to combine samples collected at various times from the same location. Sample sizes in early collections were limited because hatchlings of these endangered species had to be sacrificed for genetic analyses. With advances in techniques that allowed genetic sequences to be obtained from blood samples and, later, even less invasive skin samples, larger sample sizes have been possible (FitzSimmons et al. 1999). In addition, recent advances in statistical analyses for mixed stock assessments (Bolker et al. 2003; Okuyama and Bolker 2005) have emphasized the need for larger sample sizes. The lack of structuring within the Tortuguero population, either temporal or spatial, supports the current practice of combining samples over the geographic and temporal range of each rookery, as long as repeated sampling of any individual turtle is avoided.

For the Tortuguero population, the original sample of 15 (one egg from each of 15 females) was collected in 1988. In 1996, an additional 26 blood samples were collected from nesting females (Lahanas et al. 1998). We have now collected skin samples from 392 nesting females in 2001 and 2002 (Table 1). What would be the best sample to represent the Tortuguero population in future analyses? The probability that an individual female was resampled among the three sampling periods is extremely small because all turtles were double-tagged, survival probability between sampling periods is relatively low [annual mortality of adult females is estimated as 0.18 and 0.20 (Solow et al. 2002; Campbell 2003, respectively)], and the proportion of sampled turtles is extremely small [15 and 26 out of approximately 10,000

to 30,000 turtles in 1988 and 1996, respectively (Bjorndal et al. 1999; Troëng and Rankin 2005)]. Therefore, we believe the “combined” sample ($n=433$) is a valid sample.

Genetic diversity

With the larger sample size and the sampling protocol designed to capture temporal and spatial variation in genetic diversity, we have probably captured the majority of the mtDNA genetic diversity in the Tortuguero colony. Additional sampling may well reveal new, rare haplotypes, but substantial changes are unlikely. What proportion of genetic diversity in green turtle populations in the Greater Caribbean would be protected if all Atlantic rookeries except the Tortuguero nesting colony became extinct? The simplest measure of the proportion of genetic diversity contained in the Tortuguero rookery is the proportion of haplotypes. Fourteen haplotypes have been reported from green turtle rookeries in the Greater Caribbean (Fig. 1); this total includes the 13 haplotypes from the Greater Caribbean sample (Table 1) and CM-A27 reported from Cuba. Fifteen additional haplotypes have been reported from green turtles on foraging grounds in the Greater Caribbean, but not from Greater Caribbean rookeries (either the source rookery is not known or is outside of the Greater Caribbean). By this measure, Tortuguero would only preserve 36% of the known rookery haplotypes (5 of 14) or 17% (5 of 29) of the haplotypes known to occur in the Greater Caribbean.

Haplotype and nucleotide diversities are substantially lower for the combined Tortuguero sample than for the Greater Caribbean. From values in Table 1, Tortuguero represents 37% and 43% of Greater Caribbean rookery haplotype and nucleotide diversities, respectively. However, these values can be misleading because regional diversity values are not an additive function of individual rookery values, largely because evenness of distribution of haplotypes has a significant effect on both measures of diversity. For example, when a regional sample of rookery haplotypes is created by combining a series of haplotype frequencies from individual rookery samples, changes in the evenness of distribution among haplotypes can result in estimates of regional haplotype diversity and nucleotide diversity that are lower than those of individual rookeries.

Based on the above measures, the Tortuguero rookery captures only part of the regional diversity in mtDNA. Although conserving the Tortuguero rookery is essential to maintain abundance of green turtles in the Greater Caribbean, conserving the smaller rookeries is necessary to maintain genetic diversity in Caribbean green turtles. Patterns of genetic diversity among Caribbean green turtle rookeries should be further evaluated based on nuclear DNA.

Structure within the Tortuguero rookery

Knowledge of the extent of structuring within a rookery, either on a temporal or spatial basis, can provide insights on the mechanisms of nesting beach fidelity, site-fixity within that nesting beach, and nest site selection by female green turtles. Information on structuring is also important for management programs that strive to maintain genetic diversity. We found no temporal structuring between years or within 2002 based on mtDNA haplotypes. Hatase et al. (2002) also found no significant difference in mtDNA haplotype frequencies among years within four nesting colonies of loggerhead sea turtles in Japan. We do not know of any studies that have addressed temporal structuring within a nesting season. Of course, future studies, employing techniques that can distinguish differences at finer scales, may reveal temporal structuring among Tortuguero green turtles.

An earlier study, employing minisatellite DNA fingerprinting, provisionally indicated genetic spatial structure over 8 km at Tortuguero (Peare and Parker 1996). Relatedness of females decreased with increasing distance on the northernmost 8 km at Tortuguero. However, Peare and Parker (1996) found no relationship between relatedness and distance in green turtles nesting on 16 km of Melbourne Beach, Florida. Moore and Ball (2002) found no genetic structure in loggerheads nesting on 8 km of Melbourne Beach, Florida, with microsatellites. Genetic structure was detectable at distances of ~100 km in loggerheads nesting in the Mediterranean based on RAPD analyses of nDNA (Schroth et al. 1996) and in Florida based on mtDNA sequences (Pearce 2001). Green turtles exhibit greater site-fixity than loggerheads; that is, individual green turtles tend to deposit successive clutches within a narrower geographic range than do loggerheads (Miller 1997). Therefore, greater spatial structure would be expected to occur in green turtles compared with loggerheads. However, we found no evidence of spatial structure, at the level detectable by mtDNA haplotypes, along the 30 km of Tortuguero Beach. Further studies with microsatellite loci might be informative.

Structure among rookeries

On the basis of mtDNA restricted fragment length polymorphism (RFLP) data, Bowen et al. (1992) concluded that green turtle nesting aggregations exhibit significant, but shallow, geographic structure within ocean basins. Using mtDNA sequences, Lahanas et al. (1994) proposed a division between green turtle rookeries in the western Caribbean (Costa Rica and Florida) and eastern Caribbean (Suriname and Aves Island). Following a greater sampling of Atlantic green turtle rookeries, Encalada et al. (1996) concluded that “two distinctive evolutionary lineages were observed corresponding to the western Caribbean and Mediterranean rookeries (Cluster A) and the eastern Carib-

bean, South Atlantic and West African rookeries (Cluster B).” Clusters A and B are indicated in Fig. 1. However, the existence of these two evolutionary lineages within Atlantic green turtles has been challenged; population aggregation analysis and cladistic haplotype aggregation do not support two distinct lineages (Brower 1999).

As sample sizes have increased, the concordance between the phylogenetic clustering of haplotypes (Clusters A and B) and distinct geographical regions proposed by Encalada et al. (1996) has weakened. Compared with values from the previous Tortuguero sample, the combined Tortuguero sample yields a lower estimate of genetic structuring (Φ_{st}) among the five Caribbean rookeries, lower pairwise F_{st} values, and higher estimates of gene flow between the eastern rookeries (Aves and Surinam), although all differences are small. In the mtDNA sequences analyzed by Encalada et al. (1996), only two turtles violated the West Caribbean/Cluster A and East Caribbean/Cluster B pattern. Two additional turtles from the East Caribbean with Cluster A haplotypes were reported in Lahanas et al. (1998). Our data from Tortuguero increase the extent of shared haplotypes across the proposed geographic division. The Combined sample from Tortuguero has 37 individuals (8.5%) from Cluster B. A pattern is emerging in Greater Caribbean rookeries of two common, widely distributed haplotypes (CM-A3 and CM-A5) with the former more common in rookeries in the west and declining eastward and the latter exhibiting the opposite pattern (Fig. 2a). This pattern is similar to that exhibited in loggerheads in the southeast US in which the frequency of two common haplotypes (CC-A1 and CC-A2) are inversely related in rookeries along a north-south axis (Bowen 2003). The same inverse relationship is found in foraging aggregations of immature loggerheads along the US coast, apparently as a result of natal homing in which juvenile loggerheads return preferentially to the vicinity of their natal rookery (Bowen et al. 2004). The foraging aggregations of green turtles that have been evaluated in the Greater Caribbean exhibit a pattern similar to, but weaker than, that of green turtle rookeries (Fig. 2b), suggesting that juvenile green turtles may also display natal homing. Genetic compositions of more green turtle foraging aggregations in the Greater Caribbean are needed to evaluate these patterns.

Mixed stock analyses

Mixed stock analyses are an important tool for addressing basic ecological questions such as elucidating patterns of dispersal of sea turtles across ocean basins and understanding the extent of mixing of turtles from the different rookeries on foraging grounds (Broderick et al. 1994; Bolten et al. 1998; Lahanas et al. 1998; Bass and Witzell 2000). Mixed stock analyses are also critical for addressing management issues, such as identifying which rookeries are affected by directed take (Bass et al.

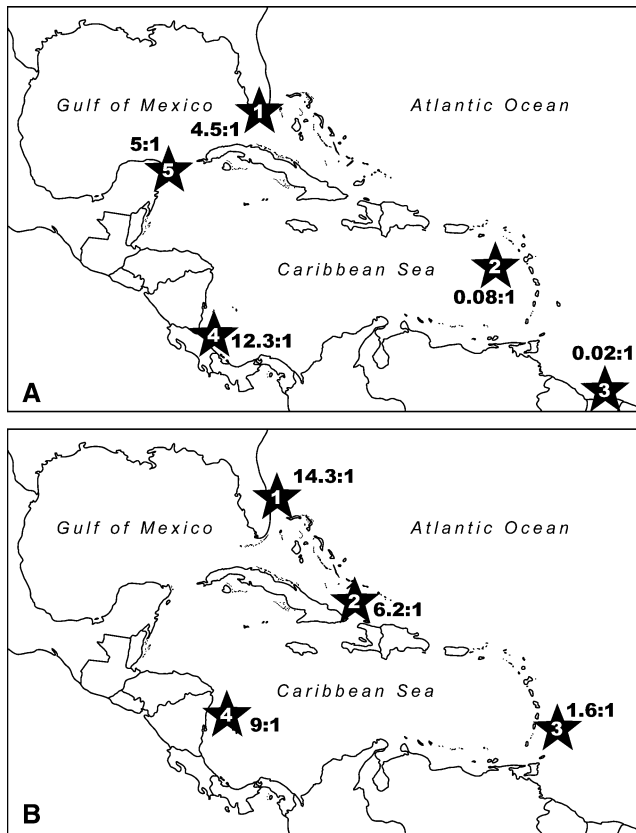


Fig. 2 Maps showing the general decline from west to east in the Greater Caribbean in the ratios of Haplotype CM-A3 to Haplotype CM-A5 in **a** green turtle rookeries: 1 Florida, USA ($n=36$, Encalada et al. 1996, Bjorndal and Bolten unpubl. data); 2 Aves Island, Venezuela ($n=55$, Lahanas et al. 1998, Bjorndal and Bolten unpubl. data); 3 Suriname ($n=46$, Encalada et al. 1996 and Bjorndal and Bolten unpubl. data); 4 Tortuguero, Costa Rica ($n=433$, this study); 5 Quintano Roo, México ($n=20$, Encalada et al. 1996); and **b** green turtle foraging aggregations: 1 Florida, USA ($n=62$, Bass and Witzell 2000); 2 Inagua, Bahamas ($n=80$, Lahanas et al. 1998); 3 Barbados ($n=60$, Luke et al. 2003); 4 Nicaragua ($n=60$; Bass et al. 1998)

1998, Díaz-Fernández et al. 1999) or incidental capture of sea turtles in commercial fisheries (Laurent et al. 1998).

The larger sample for Tortuguero green turtles presented here has a substantial effect on mixed stock analyses in the Atlantic. First, two haplotypes (CM-A20 and CM-A21) that had previously only been reported from a foraging ground in the southern Bahamas (Lahanas et al. 1998) can now be assigned to the Tortuguero rookery. Second, changing the proportion of haplotype CM-A5 at Tortuguero from 0 to 7.4% (in the Combined sample, Table 1) may significantly alter the results of mixed stock analyses. For example, in a mixed stock analysis of adult green turtles from a foraging ground off the east coast of Nicaragua (Bass et al. 1998), a sample of 60 adult green turtles yielded 54 CM-A3 and 6 CM-A5; the 30 adult females in the sample yielded 29 CM-A3 and 1 CM-A5. Bass et al. (1998) reported that chi-square analyses indicated that haplotype frequency of the foraging ground sample differed

significantly from those of each of the rookeries, and subsequent mixed stock analyses assigned Tortuguero and Aves Island/Suriname as contributing rookeries. However, with the combined Tortuguero sample, the haplotype frequencies of the foraging ground sample are not significantly different from that of the Tortuguero rookery (all adults: $\chi^2=1.306$, $df=4$, $p=0.815$; females only: $\chi^2=1.154$, $df=4$, $p=0.653$). Therefore, based on genetic evidence, the Nicaraguan foraging aggregation would not be considered a mixed stock; the most parsimonious conclusion is that Tortuguero is the sole contributing rookery. However, based on flipper tags, we know that some females that nested on Aves Island have been captured on foraging grounds off the east coast of Nicaragua (Carr et al. 1978). A larger sample of mtDNA sequences from the Nicaraguan foraging grounds would allow more accurate assessment of source rookeries. We compared the haplotype frequencies of three other foraging ground samples [southern Bahamas (Lahanas et al. 1998), Florida (Bass and Witzell 2000), and Barbados (Luke et al. 2004)] with the current Tortuguero sample and found that they were still significantly different from that of the Tortuguero rookery (Bahamas: $\chi^2=31.5$, $df=7$, $p<0.0001$; Florida: $\chi^2=116.0$, $df=8$, $p<0.0001$; Barbados: $\chi^2=220.0$, $df=10$, $p<0.0001$).

Mixed stock analyses are underway in a number of laboratories, and improved statistical approaches are now available for mixed stock analyses (Bolker et al. 2003; Okuyama and Bolker 2005). Expanded samples from foraging grounds and rookeries, such as the one presented here for Tortuguero, are essential for the accurate evaluation of the phylogeography and dispersal patterns in Atlantic green turtles.

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