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Mitogenomic sequences better resolve stock structure of southern Greater Caribbean green turtle rookeries

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Abstract

Analyses of mitochondrial control region polymorphisms have supported the presence of several demographically independent green turtle (*Chelonia mydas*) rookeries in the Greater Caribbean region. However, extensive sharing of common haplotypes based on 490-bp control region sequences confounds assessment of the scale of natal homing and population structure among regional rookeries. We screened the majority of the mitochondrial genomes of 20 green turtles carrying the common haplotype CM-A5 and representing the rookeries of Buck Island, St. Croix, United States Virgin Islands (USVI); Aves Island, Venezuela; Galibi, Suriname; and Tortuguero, Costa Rica. Five single-nucleotide polymorphisms (SNPs) were identified that subdivided CM-A5 among regions. Mitogenomic pairwise ϕ_{ST} values of eastern Caribbean rookery comparisons were markedly lower than the respective pairwise F_{ST} values. This discrepancy results from the presence of haplotypes representing two divergent lineages in each rookery, highlighting the importance of choosing the appropriate test statistic for addressing the study question. Haplotype frequency differentiation supports demographic independence of Aves Island and Suriname, emphasizing the need to recognize the smaller Aves rookery as a distinct management unit. Aves Island and Buck Island rookeries shared mitogenomic haplotypes; however, frequency divergence suggests that the Buck Island rookery is sufficiently demographically isolated to warrant management unit status for the USVI rookeries. Given that haplotype sharing among rookeries is common in marine turtles with cosmopolitan distributions, mitogenomic sequencing may enhance inferences of population structure and phylogeography, as well as improve the resolution of mixed stock analyses aimed at estimating natal origins of foraging turtles.

Keywords: *Chelonia mydas*, Greater Caribbean, green turtle, haplotype, mitogenome, population structure

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Introduction

Defining population structure of highly vagile marine species can be challenging given their ability to disperse over vast spatial scales. For marine turtles, population boundaries have been typically delimited on the basis of female philopatry to natal rookeries (Bowen *et al.* 1992, 1993a; Norman *et al.* 1994). It is therefore critical from a conservation perspective to properly characterize the scale of this natal homing behaviour to ensure that demographically isolated rookeries receive adequate recognition and protection. Several marine turtle species, including green turtles (*Chelonia mydas*), share similar complex life histories with respect to developmental and seasonal migrations (Musick & Limpus 1997; Bolten 2003). The first step of the life cycle involves an oceanic juvenile dispersal stage that sometimes encompasses

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entire ocean basin gyres (Bowen *et al.* 1995; Bolten *et al.* 1998; Monzón-Argüello *et al.* 2010a). Following the oceanic stage, turtles often recruit to neritic foraging areas (Musick & Limpus 1997; Bolten 2003), where juvenile and adult foraging aggregations are usually comprised of individuals from multiple nesting populations (reviewed in Bowen & Karl 2007). As these foraging grounds are often not proximal to nesting beaches, adult turtles make seasonal shuttling migrations between foraging grounds and breeding grounds adjacent to nesting beaches during their reproductive years (Carr *et al.* 1978). Characterizing the migratory connectivity of source rookeries and foraging aggregations is an important conservation consideration (Harrison & Bjorndal 2006), given the need to protect highly migratory species throughout their life cycle (Martin *et al.* 2007).

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

Although female natal philopatry appears to be the primary force shaping population structure of green turtle rookeries within ocean basins (Bowen *et al.* 1992), this homing behaviour must occasionally break down to permit the colonization of novel nesting habitats (Carr *et al.* 1978). Combined with slow evolution of the mitochondrial genome of marine turtles relative to many other vertebrates (Avise *et al.* 1992; Bowen *et al.* 1993b), this ability to colonize sites distant from their natal regions has lead to extensive haplotype sharing (based on 390-bp to 500-bp control region sequences) among rookeries over large spatial scales in all marine turtles species with cosmopolitan distributions (reviewed in Bowen & Karl 2007). Among Atlantic green turtle rookeries, the majority of sampled individuals carried four common 490-bp control region haplotypes belonging to two shallow lineages (CM-A1, CM-A3, CM-A5 and CM-A8; Fig. 1; (Encalada *et al.* 1996; Lahanas *et al.* 1998; Bjorndal *et al.* 2005, 2006; Formia *et al.* 2006, 2007). In the Greater Caribbean region, CM-A5 is the most common haplotype at Suriname and Aves Island and is the second most common haplotype at Tortuguero, Costa Rica (Encalada *et al.* 1996; Lahanas *et al.* 1998; Bjorndal *et al.* 2005). Despite approximately 1300 km of separation, the rookeries of Matapica, Suriname and Aves Island were not significantly different with respect to their 490-bp haplotype frequencies,

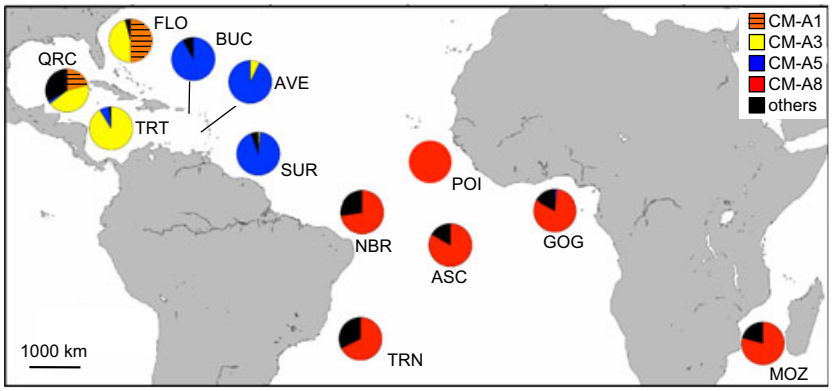

Fig. 1 Locations and frequency distribution of common 490-bp mitochondrial control region haplotypes for select Atlantic and southwest Indian Ocean green turtle rookeries. Some distinct management units were combined for legibility. FLO, Florida, United States of America (Encalada *et al.* 1996); QRC, combined Quintana Roo and southwestern Cuba (Encalada *et al.* 1996, Ruiz-Urquiola *et al.* 2010); TRT, Tortuguero, Costa Rica (Encalada *et al.* 1996; Bjorndal *et al.* 2005); BUC, Buck Island, United States Virgin Islands (present study); AVE, Aves Island, Venezuela (Encalada *et al.* 1996; Lahanas *et al.* 1998; present study); SUR, combined Matapica and Galibi, Suriname (Encalada *et al.* 1996; present study); POI, Poilão, Guinea Bissau (Formia *et al.* 2006); NBR, combined Atol das Rocas and Fernando de Noronha (Encalada *et al.* 1996; Bjorndal *et al.* 2006); ASC, Ascension Island (Encalada *et al.* 1996; Formia *et al.* 2006, 2007); GOG, combined Bioko and Corisco, Equatorial Guinea as well as São Tomé and Principe (Formia *et al.* 2006); TRN, Trindade Island, Brazil (Bjorndal *et al.* 2006); and MOZ, combined Juan de Nova and Europa (Bourjea *et al.* 2007).

although with the caveat that this was likely due to recent isolation rather than contemporary exchange of females between rookeries (Encalada *et al.* 1996). Discerning between these alternative scenarios is important for assessing population structure for management on ecological timescales. Moreover, the overlap of genetic markers among rookeries has the potential to introduce considerable uncertainty into estimates of rookery contributions to mixed foraging aggregations (Bolker *et al.* 2007), even if it is clear that the rookeries are demographically partitioned based on haplotype frequency differences. Estimates based on carrying capacity of seagrass pastures, historic nesting population descriptions and harvest records suggest that the contemporary abundance of green turtles in the Caribbean is less than 7% of pre-Columbian numbers (Jackson *et al.* 2001; McClenachan *et al.* 2006). This decline highlights the importance of assessments of population structure and migratory connectivity of breeding and developmental habitats in the region.

Novel genetic data may resolve extensive sharing of common haplotypes based on short fragments of the mitochondrial control region. Some nuclear markers offer a quickly evolving alternative to mitochondrial DNA. However, nuclear surveys have generally detected equivalent or considerably less structure than that inferred using mitochondrial markers at regional spatial scales (hundreds of km; Karl *et al.* 1992; FitzSimmons *et al.* 1997b; Roberts *et al.* 2004; Bowen *et al.* 2005; but see Carreras *et al.* 2007). Genetic surveys based on nuclear markers (RFLP analysis of anonymous single-copy loci and four microsatellites) failed to detect differentiation among Greater Caribbean green turtle rookeries (Karl *et al.* 1992; Roberts *et al.* 2004; Wallace *et al.* 2010), despite marked mtDNA haplotype frequency differences among several rookeries in the region (Encalada *et al.* 1996). This disparity in signal has been attributed, at least in part, to male-mediated or migration-mediated nuclear gene flow in the presence of strong natal philopatry by females (Karl *et al.* 1992; FitzSimmons *et al.* 1997b). Therefore, expanded screening of the mitochondrial genome may benefit analyses of genetic structure among populations at regional spatial scales where nuclear gene flow is likely to occur via population admixture on foraging grounds or along migratory corridors. MtDNA analysis beyond established control region fragments has improved the resolution of intraspecific phylogeography and population structure of several marine taxa. Haplotype CC-A1, based on 390 bp of the control region, that is shared between western Atlantic and Cape Verde (eastern Atlantic) loggerhead turtle rookeries has been subdivided into apparently endemic haplotypes through comparisons of an expanded 760-bp control region fragment (Monzón-Argüello *et al.* 2010b). Phylogeographic analysis of whole mitochondrial genome sequence variation in killer whales (*Orcinus orca*) provided strong support for species status of the ecotypes (Morin *et al.* 2010), whereas an earlier analysis based on shorter sequences failed to resolve these relationships because of the limited polymorphism (Hoelzel *et al.* 2002). Despite sharing haplotype CM-A8 with Brazilian and Ascension rookeries (Encalada *et al.* 1996), green turtles from Guinea Bissau carried a unique restriction digest profile at a *DraII* site (Bowen *et al.* 1992), illustrating that additional informative variation occurs outside the established control region fragment.

To date, marine turtle haplotypes have been assigned based on <1 kilobase (kb) of the >16-kb mitogenome. Undescribed polymorphism outside the established control region fragments may remedy several intractable cases of haplotype overlap among marine turtle rookeries and improve the resolution of population structure as well as mixed stock analyses. We sequenced the majority of the mitochondrial genome (16350 of 16497 bp, 99%) of 20 nesting turtles with control region haplotype CM-A5 from the rookeries of Buck Island, USVI; Aves Island, Venezuela; Galibi, Suriname; and Tortuguero, Costa Rica to identify the informative single-nucleotide polymorphisms (SNPs). Genetic characterization of the Buck Island rookery to assess its relationship with the Aves Island rookery and reanalysis of population structure among four southern Greater Caribbean rookeries using novel mitogenomic sequence variation were the objectives of this study.

Methods

The samples sequenced in this study were collected at four green turtle rookeries in the southern Greater Caribbean region: Tortuguero, Costa Rica; Buck Island, USVI; Aves Island, Venezuela; and Galibi, Suriname (Fig. 2). Samples previously analysed for the standard 490-bp control region haplotypes include all CM-A5, CM-A20 and CM-A21 individuals sampled at Tortuguero in 2001 and 2002 (n = 37; Bjorndal *et al.* 2005); Aves Island samples from four previous studies (n = 25, 4, 4, and 34, respectively; Bowen *et al.* 1992; Lahanas *et al.* 1998; Roberts *et al.* 2004; unpublished data in Bolker *et al.* 2007) and Galibi, Suriname individuals sampled in 1999 and 2000 (n = 58; unpublished data in Bolker *et al.* 2007). Additional samples were collected on Buck Island from females nesting from 2001 through 2009 (n = 49). Skin biopsies were collected using 6-mm biopsy punches and stored in a 20% DMSO-saturated NaCl buffer (Dutton & Balazs 1995). Each female was tagged with Inconel tags in both front flippers (Balazs 1999) to ensure that individuals were sampled only once.

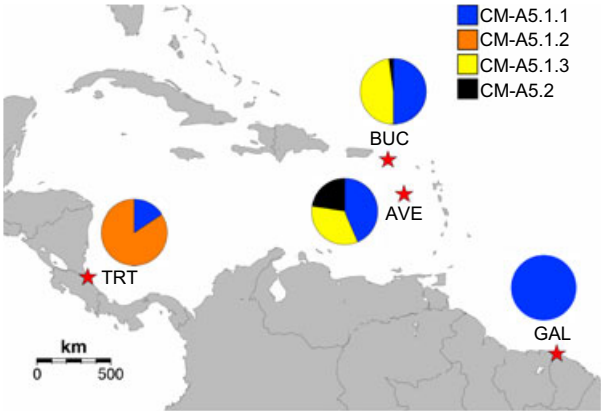

Fig. 2 Locations and frequencies of mitogenomic CM-A5 haplotypes for select green turtle rookeries in the southern Greater Caribbean region: TRT, Tortuguero, Costa Rica; BUC, Buck Island, St. Croix, United States Virgin Islands; AVE, Aves Island, Venezuela; and GAL, Galibi, Suriname.

PCR for control region amplification was carried out in 20 μL volumes using primers LCM15382 (GCTTA ACCCTAAAGCATTGG; Abreu-Grobois *et al.* 2006) and a novel reverse primer CM16437 (TTGGTTGAGG TGTGGTAGAG). The novel primer was designed to amplify an additional 150 bases beyond the fragment amplified by LCM15382 and the reverse primer H950g (Abreu-Grobois *et al.* 2006), and extends the fragment to just 5′ of the repetitive element in the control region. Additional portions of the mitogenome (the complete genome less the Phe-tRNA and the repetitive element in the control region) were amplified in 25 μL volumes using primers designed from the published green turtle mitochondrial genome (Table S1, Supporting information; Kumazawa & Nishida 1999; GenBank AB012104). Reactions contained 10 mM Tris, pH 8.4; 50 mM KCl; 0.5 μM of each primer; 1.5 mM $MgCl_2$; 0.25 mM dNTPs; 1.0 unit of *Taq* DNA polymerase; and approximately 10 ng of genomic DNA. PCR cycling parameters were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60–90 s depending on fragment length; and a final extension of 72 °C for 10 min. PCR products were purified using ExoSAP-IT® (USB Corporation) according to the manufacturer's instructions. The control region amplicons were sequenced in a single direction with LCM15382 and an internal forward sequencing primer (Cm15821, TCACGAGAAATAAG-CAAC) using ABI BigDye v3.1 and an ABI 3730*xl* DNA Analyzer (PE Applied Biosystems). Sequencing reactions for additional portions of the mitochondrial genome were conducted in a single direction using forward PCR primers as well as internal sequencing primers designed from the published green turtle mitochondrial genome (Table S1, Supporting information). Negative controls were included with PCR amplification and sequencing reactions to detect any potential contamination. The initial round of mitochondrial genome screening was performed using two CM-A5 individuals each from the rookeries of Galibi, Aves Island and Tortuguero and two CM-A3 individuals from Tortuguero for comparative purposes. Following mitogenomic haplotype assignments based on the SNPs detected in the first round of screening, the mitochondrial genomes of 14 additional CM-A5 turtles representing Galibi (n = 5), Aves Island (n = 4), Buck Island (n = 4) and Tortuguero (n = 1) were sequenced in search of further polymorphism. CM-A6, CM-A20 and CM-A21 were screened only at the SNPs identified for CM-A5 and CM-A3.

Sequences were aligned, edited and compared to previously described haplotypes and the published green turtle mitochondrial genome using the program Sequencher 4.2 (Gene Codes Corporation). Sequences were assigned haplotype designations after nomenclature published on the Archie Carr Center for Sea Turtle Research (ACCSTR) website (http://accstr.ufl.edu/cmmtdna.html). Mitogenomic haplotype names consist of a series of three numerals corresponding to different fragments of the mitochondrial genome. The first number in the series denotes the original haplotype name based on a approximately 490-bp fragment of the mtDNA control region. The second number in the series denotes the variants based on polymorphisms within the 817-bp control region fragment amplified by LCM15382-H950 (but outside the original 490-bp fragment) that subdivided the original haplotype. Finally, the third number represents the variants defined by polymorphisms outside the 817-bp control region fragment. Samples producing novel or ambiguous sequences were subjected to a second round of DNA extraction, PCR amplification and sequencing for verification. Novel haplotypes were deposited with GenBank and ACCSTR. Maps were created using the Maptool function (SEATURTLE.ORG Maptool 2002).

Haplotype frequency–based pairwise F_{ST} comparisons, distance-based pairwise ϕ_{ST} comparisons, pairwise exact tests of population differentiation and haplotype frequency– and distance-based analysis of molecular variance (amova) were conducted using the software Arlequin version 3.1 (Excoffier *et al.* 2005). Sequence divergence estimates were generated using the Tamura–Nei model (Tamura & Nei 1993). Significance values for amova were obtained from 10 000 permutations. Exact tests of population differentiation were conducted with 100 000 permutations and 10 000 dememorization steps after the method of Raymond & Rousset (1995). All analyses were conducted using the short haplotypes based on a 490-bp fragment of the 5′ end of the control region (Allard *et al.* 1994), the long haplotypes based on an 817-bp fragment of the control region (Abreu-Grobois *et al.* 2006), as well as the expanded mitogenomic

haplotypes identified in the present study. Significance of all pairwise comparisons was adjusted using sequential Bonferroni correction (Rice 1989). We used POWSIM (Ryman & Palm 2006) to assess the relative power of haplotypes based on each of the three fragments for detecting population structure. Power analyses were conducted to determine the minimum sample size per rookery needed to detect the lowest F_{ST} value generated from the analysis of mitogenomic haplotype frequencies with 95% probability. Empirical aggregate haplotype frequencies for the three eastern Caribbean rookeries were used as the starting frequencies of the base population prior to implementing genetic drift.

Results

Based on the 490-bp fragment, two haplotypes were detected at the Buck Island rookery: CM-A5 (n = 45, 92%) and CM-A16 (n = 4, 8%). Sequence alignments of the LCM15382 and CM16437 control region amplicon (approximately 1050 bp) revealed two additional polymorphic sites outside the established 490-bp control region fragment: an indel within the CM-A5 lineage and a variable position between the haplogroups containing CM-A3 and CM-A5 (Table 1). The conserved haplotype has been designated CM-A5.1, and the haplotype with the insertion has been designated CM-A5.2. Both polymorphic sites fell within the LCM15382 and H950 amplicon; therefore, no additional polymorphism was detected through the use of the CM16437 reverse primer. Mitogenomic sequence alignments from outside the control region revealed four variable positions corresponding to three mitogenomic CM-A5.1 haplotype variants: CM-A5.1.1, CM-A5.1.2 and CM-A5.1.3 (Table 1). Control region haplotypes CM-A20.1 and CM-A21.1 from Tortuguero shared the two derived mutations present in the common variant CM-A5.1.2 present at Tortuguero (Fig. 3). In addition to the polymorphisms detected within the CM-A5 lineage, mitochondrial genome sequence alignments from outside

Table 1 Variable positions for southern Greater Caribbean nesting green turtles based on CM-A5 and CM-A3 mitochondrial genome sequencing. Numbers correspond to base locations in the established control region alignments and the published mitochondrial genome

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

	490 bp	137	161	238	260	272	353	355	398	419	421	475		
	817 bp	216	240	317	339	351	432	434	477	498	500	554	682	806
Haplotype	mtgenome	15664	15688	15765	15787	15799	15880	15882	15925	15946	15948	16002	16130	16254
CM-A5.1.1		G	A	C	T	G	C	A	C	G	A	G	T	–
CM-A5.1.2		.	.	.	.	.	.	.	.	.	.	.	.	–
CM-A5.1.3		.	.	.	.	.	.	.	.	.	.	.	.	–
CM-A5.2		.	.	.	.	.	.	.	.	.	.	.	.	C
CM-A6.1		.	.	.	.	.	.	.	.	.	.	A	.	–
CM-A20.1		.	G	.	.	.	.	.	.	.	.	.	.	–
CM-A21.1		.	.	.	.	.	.	.	T	.	.	.	.	–
CM-A3.1.1		.	.	T	.	T	.	G	T	A	G	A	C	–
CM-A16.1		A	.	.	C	T	T	G	T	A	G	A	C	–
CM-A4		.	.	T	.	T	T	G	T	A	G	A	NA	NA

NA indicates positions that were not analysed.
The light gray shading indicates variable positions outside the control region. The dark gray shading indicates novel control region variation found using primers LCM15382 and CM16437.

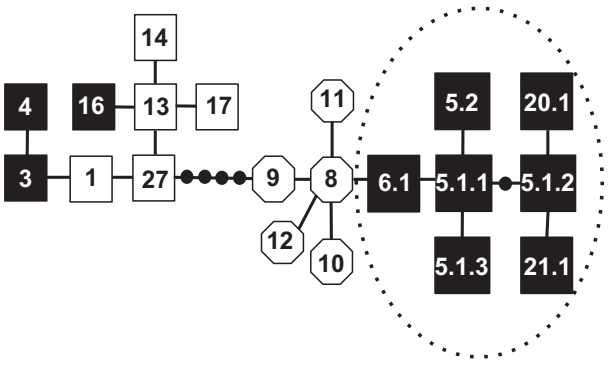

Fig. 3 Haplotype network modified from Bjorndal *et al*. (2005) illustrating common Atlantic green turtle control region haplotypes based on the 490-bp fragment. Haplotypes encircled by the dotted line illustrate mitogenomic variation within the CM-A5 lineage. Greater Caribbean and Mediterranean haplotypes are in squares; equatorial Atlantic haplotypes are in hexagons. Filled circles represent hypothetical haplotypes. Haplotypes in black were identified at the four rookeries in the present study. 'CM-A' prefixes are excluded for legibility.

the control region identified seven SNPs that were variable between CM-A5.1.1 and the two CM-A3.1.1 nesting females from Tortuguero. The second round of mitogenomic screening did not detect any additional SNPs.

Novel polymorphism identified in this study was highly informative with respect to regional population structure. The conserved mitogenomic variant CM-A5.1.1 was found in all four rookeries surveyed and was the only CM-A5 variant recorded at Galibi, Suriname (Fig. 2; Table 2). CM-A5.1.2 occurred at high frequency at Tortuguero (84% of CM-A5 variants) but was not found elsewhere. CM-A5.1.3 was common at both Aves Island (31%) and Buck Island (43%) but absent at Galibi and Tortuguero. CM-A5.2.1 was common in the Aves Island rookery (21%) but detected in only a single nesting female at Buck Island and was not found among Galibi or Tortuguero females.

The overall structure partitioned among the four rookeries by AMOVA was high across all sequence lengths analysed (Table 3). However, AMOVA of the three eastern Caribbean rookeries detected differences only when 817-bp and mitogenomic haplotypes were considered (Table 3). All Tortuguero vs. eastern Greater Caribbean pairwise comparisons were significantly different across all haplotype lengths and all test statistics (Table 4). However, none of the short haplotype eastern Caribbean comparisons were significant with respect to pairwise F_{ST} values or ϕ_{ST} values, and Aves and Galibi were not differentiated based on pairwise exact tests of population differentiation when only the 490-bp haplotypes were analysed (Table 4). Frequency analysis of 817-bp control region haplotypes yielded additional significant differences between Buck Island and Galibi as well as between Aves Island and Galibi. Among mitogenomic comparisons, only the Aves Island and Buck Island genetic distance–based test was insignificant, despite a signal of differentiation with frequency-based tests (Table 4). Power analyses indicated that the sample sizes needed to detect F_{ST} = 0.031 with 95% probability for 490-, 817-bp and mitogenomic haplotypes were 115, 80 and 58, respectively. The former sample size is larger than the estimated size of the female population currently nesting on Buck Island (Buck Island Reef National Monument, unpublished data). Reaching this sample size would have required sampling approximately 16–38% of females nesting annually on Aves Island based on tagging data from the previous two decades (Buitrago *et al*. 2008).

Table 2 Mitogenomic haplotype frequencies for southern Greater Caribbean green turtle rookeries. CM-A3.X and CM-A4 counts represent published data based on 490-bp mitochondrial control region sequences only (Bjorndal *et al*. 2005; Encalada *et al*. 1996)

	Tortuguero	Buck Island	Aves Island	Galibi
CM-A3.X	393			
CM-A3.1	2		5	1
CM-A4	1			
CM-A5.1.1	5	23	27	55
CM-A5.1.2	27			
CM-A5.1.3		21	21	
CM-A5.2		1	14	
CM-A6.1				2
CM-A16.1		4		
CM-A20.1	2			
CM-A21.1	3			

Discussion

Population structure

Comparative mitogenomic analysis revealed that 490-bp haplotype CM-A5 is an assemblage of at least four

Table 3 AMOVA results for frequency- and distance-based comparisons of all rookeries and just the three eastern Caribbean rookeries

	F_{ST}	*P*	φ_{ST}	*P*
All rookeries				
490 bp	0.812	<0.00001	0.820	<0.00001
817 bp	0.765	<0.00001	0.820	<0.00001
Mitogenome	0.700	<0.00001	0.811	<0.00001
Eastern rookeries				
490 bp	0.015	0.10386	0.012	0.17782
817 bp	0.104	<0.00001	0.011	0.17960
Mitogenome	0.239	<0.00001	0.051	0.00436

Table 4 Pairwise F_{ST} values (above the diagonal), pairwise φ_{ST} values (below the diagonal) and *P* values from exact tests of population differentiation (bottom table) between southern Greater Caribbean green turtle rookeries. Values were generated from 490-bp control region, 817-bp control region and mitogenomic comparisons, respectively. *F_{ST} and φ_{ST} comparisons significant at $\alpha = 0.05$ with sequential Bonferroni correction for multiple tests

	Tortuguero	Buck Island	Aves Island	Galibi
Tortuguero		0.827*, 0.821*, 0.748*	0.817*, 0.743*, 0.679*	0.834*, 0.834*, 0.843*
Buck Island	0.822*, 0.822*, 0.816*		0.024, 0.103*, 0.031*	0.017, 0.020, 0.382*
Aves Island	0.820*, 0.820*, 0.806*	−0.001, −0.002, −0.002		0.004, 0.140*, 0.337*
Galibi	0.848*, 0.848*, 0.837*	0.026, 0.026, 0.136*	0.016, 0.016, 0.058*	
	Tortuguero	Buck Island	Aves Island	Galibi
Tortuguero		<0.00001, <0.00001, <0.00001	<0.00001, <0.00001, <0.00001	<0.00001, <0.00001, <0.00001
Buck Island			0.00541, 0.00033, 0.00020	0.02091, 0.01635, <0.00001
Aves Island				0.09120, 0.00002, <0.00001
Galibi				

distinct lineages that are subdivided among regional rookeries. Mitogenomic haplotype frequencies were significantly different for each of the four sampled rookeries, suggesting that sufficient demographic partitioning exists to warrant separate management unit status for the Buck Island, Aves Island, Suriname and Tortuguero nesting populations. The latter has always been considered genetically distinct from eastern Caribbean rookeries based on haplotype frequency differences (Encalada *et al.* 1996), although more thorough sampling at Tortuguero revealed increased haplotype sharing with eastern Caribbean rookeries relative to the initial survey (Bjorndal *et al.* 2005). Mitogenomic analysis indicated that approximately 84% of the CM-A5 females nesting at Tortuguero belong to a lineage that was not recorded elsewhere in the Greater Caribbean region. Genetic evidence of population subdivision between Aves Island and Suriname rookeries had not been previously detected using partial control region haplotypes, although Encalada *et al.* (1996) cautioned that the lack of differentiation was likely attributable to recent isolation rather than ongoing gene flow. Mitogenomic data corroborate the hypothesis of demographic isolation of these rookeries.

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

The strong discrepancy between frequency-based F_{ST} and genetic distance–weighted ϕ_{ST} among eastern Caribbean pairwise comparisons results from the presence of individuals representing two divergent lineages (Greater Caribbean and south Atlantic clades) in each of the four sampled rookeries. This haplotype distribution creates a scenario in which interhaplotypic differences within rookeries are greater than interhaplotypic differences among rookeries, thus eroding population genetic signal. Whereas much of the diversification of the CM-A5-derived haplotypes appears to have occurred *in situ* regionally, the presence of CM-A3 and CM-A16 in eastern Caribbean rookeries likely results from relatively recent colonization from western Caribbean sources. Marine turtles have tremendous dispersal capability when natal homing breaks down, which has lead to the colonization of the same nesting beach by turtles representing divergent lineages (Bowen *et al.* 1993a; Bjorndal *et al.* 2005; Dethmers *et al.* 2006; Bourjea

et al. 2007; Browne *et al.* 2010; present study). In these cases, ϕ_{ST} values may be biased downward, whereas frequency-based measures will better reflect demographic partitioning, as has been previously noted for cetacean populations with similar haplotype distributions and phylogeographic structure (O'Corry-Crowe *et al.* 1997; Rosel *et al.* 1999). Use of distance-based analyses under these circumstances would conceal rather than detect population structure; therefore, the choice of a test statistic appropriate for addressing the study question is imperative (O'Corry-Crowe *et al.* 1997). A major objective of this research was to determine the female demographic connectivity among rookeries in the Greater Caribbean region, and therefore, the lack of differentiation detected between Aves Island and Buck Island rookeries with ϕ_{ST} should not detract from their recognition as distinct management units given significant haplotype frequency differentiation as measured through F_{ST} and exact tests.

Phylogeography

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

Two derived mitogenomic CM-A5 variants were present in the Aves Island and Buck Island rookeries, but both were absent among other sampled rookeries. Historical green turtle nesting in the USVI was characterized as 'minor' relative to the high nesting densities at Aves Island (McClenachan *et al.* 2006). Therefore, the two common mitogenomic lineages nesting on Buck Island (CM-A5.1.1 and CM-A5.1.3) may ultimately descend from the Aves Island population. Given the erosional nature of Aves Island (Schubert & Laredo 1984), straying may have occurred during a period of inundation when insufficient suitable nesting habitat was available. The nearest islands to Aves Island are those of the Lesser Antilles more than 175 km distant. In addition to St. Croix and Buck Island, USVI, St. Eustatius and Guadeloupe host regular green turtle nesting in low numbers (<100 crawls per beach per year; Dow *et al.* 2007). Still lower numbers of green turtle nests are recorded from several other islands of the Lesser Antillean chain. Genetic characterization of these smaller rookeries is needed to better understand the spatial and temporal scales of demographic connectivity of green turtle rookeries in the region.

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

Conservation benefits of mitogenomic population structure assessments

Several studies have demonstrated the utility of mitogenomic sequencing for resolving problematic nodes and producing more robust estimates of divergence times in a phylogenetic context (e.g. Inoue *et al.* 2001; Zhang *et al.* 2004; Pereira & Baker 2006). Mitogenomic sequencing has also proven beneficial in improving genetic signal in intraspecific phylogeographic studies (Ingman *et al.* 2000; Carr & Marshall 2008; Carr *et al.* 2008; Morin *et al.* 2010; Stone *et al.* 2010; Wang *et al.* 2010). The present study extends the utility of mitogenomic sequencing for population structure analyses in a taxon with low levels of nucleotide diversity within haplogroups and shallow evolutionary population structure within ocean basins (Bowen *et al.* 1992; Encalada *et al.* 1996). Control region haplotype sharing among rookeries is a common analytical problem in all marine turtle species with cosmopolitan distributions. Phylogeographic and population structure assessments as well

as mixed stock analyses of several marine turtle taxa could benefit from mitogenomic SNP discovery and analyses. Mitogenomic sequences of green turtle haplotype CM-A8 may hold potential for revisiting diverse questions such as determining the origin of this lineage nesting in the southwest Indian Ocean, resolving colonization sequence among south Atlantic rookeries, exploring the demographic connectivity between Ascension Island and Bioko Island rookeries and refining rookery contribution estimates to juvenile foraging aggregations across the south Atlantic. Clearly, some overlap of haplotypes among rookeries remains despite the expanded sequencing effort. This haplotype sharing may never be fully resolved given marine turtle dispersal capability and inferred slow rate of mtDNA evolution (Avise *et al*. 1992). Nonetheless, the present study demonstrates the utility of mitogenomic SNPs for detecting cryptic structure among populations that are marked by extensive sharing of a common haplotype based on <1 kb of the mitogenome. More robust data on the number of population units and the distribution of individuals representing them throughout their complex life cycle should aid management of these imperilled species.

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Data accessibility

DNA sequences: GenBank accessions JN32497–JN32505, JQ026233, JQ034420, JQ366073. See Data S1 for individual sample metadata and haplotype accession numbers.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Characterization of mitochondrial genome fragments analyzed for southern greater Caribbean green turtles and their primers.

Data S1 Individual sample metadata and haplotype accession numbers.

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