

Re-examination of Population Structure and Phylogeography of Hawksbill Turtles in the Wider Caribbean Using Longer mtDNA Sequences

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Abstract

Management of the critically endangered hawksbill turtle in the Wider Caribbean (WC) has been hampered by knowledge gaps regarding stock structure. We carried out a comprehensive stock structure re-assessment of 11 WC hawksbill rookeries using longer mtDNA sequences, larger sample sizes ($N = 647$), and additional rookeries compared to previous surveys. Additional variation detected by 740 bp sequences between populations allowed us to differentiate populations such as Barbados-Windward and Guadeloupe ($F_{st} = 0.683$, $P < 0.05$) that appeared genetically indistinguishable based on shorter 380 bp sequences. POWSIM analysis showed that longer sequences improved power to detect population structure and that when $N < 30$, increasing the variation detected was as effective in increasing power as increasing sample size. Geographic patterns of genetic variation suggest a model of periodic long-distance colonization coupled with region-wide dispersal and subsequent secondary contact within the WC. Mismatch analysis results for individual clades suggest a general population expansion in the WC following a historic bottleneck about 100 000–300 000 years ago. We estimated an effective female population size (N_{ef}) of 6000–9000 for the WC, similar to the current estimated numbers of breeding females, highlighting the importance of these regional rookeries to maintaining genetic diversity in hawksbills. Our results provide a basis for standardizing future work to 740 bp sequence reads and establish a more complete baseline for determining stock boundaries in this migratory marine species. Finally, our findings illustrate the value of maintaining an archive of specimens for re-analysis as new markers become available.

Key words: conservation genetics, *Eretmochelys imbricata*, management units, mitochondrial DNA, sea turtle, stock structure

Hawksbill turtles (*Eretmochelys imbricata*) are widely distributed throughout tropical and subtropical regions of the world, including the Wider Caribbean (WC), where their nesting distribution extends from southern Florida into the Gulf of Mexico, and south to Brazil (Meylan 1984). Their population boundaries are often difficult to define, due to

the long-distance migrations they undertake between foraging and nesting sites (Troëng et al. 2005; Vélez-Zuazo et al. 2008; Horrocks et al. 2011). Hawksbills are listed as Critically Endangered by the International Union for the Conservation of Nature, IUCN (2010) based on global population declines during the last century attributed

to direct harvest, habitat loss, and fisheries interactions, among other threats (National Marine Fisheries Service and U.S. Fish and Wildlife Service 1993; Meylan and Donnelly 1999; Bjorndal and Jackson 2003; Mortimer and Donnelly 2008).

In recent years, molecular techniques have played a significant role in informing management and defining priorities for marine species conservation (e.g., O’Corry-Crowe et al. 2006; Rosenbaum et al. 2009). Mitochondrial DNA (mtDNA) techniques, which characterize maternal lineages within and among species, have been useful in distinguishing sea turtle rookery stock structure where fixed or significant differences in haplotype frequencies occur (Bowen et al. 1992; Bass et al. 1996; Dutton et al. 1999; Shanker et al. 2004). However, when haplotypes overlap among nesting populations and their frequencies are similar, there is a danger of failing to detect an existing population differentiation (Type II error) because of insufficient resolution in the marker. This leads to lumping populations incorrectly and failing to identify appropriate units for conservation (Taylor and Dizon 1999).

Sea turtle studies have widely used mtDNA primers that amplify 384–480 base pairs (bp) of the control region (Allard et al. 1994; Norman et al. 1994). Although effective in distinguishing major rookeries over broad geographic scales in earlier genetic surveys, there is concern about the ability of these markers, particularly where haplotype diversity is low, to resolve structure at finer scales as the number of candidate rookeries increases and the amount of haplotype frequency overlap becomes greater (Abreu-Grobois et al. 2006; Vélez-Zuazo et al. 2006). The estimation of stock composition of foraging populations in the region has been problematic since numerous mtDNA haplotypes are shared by WC hawksbill rookeries (Bass et al. 1996; Bass 1999; Bowen and Karl 2007). This in turn has contributed to geopolitical controversy related to the management of this endangered species as a trans-boundary resource (Bowen et al. 2007a; Mortimer et al. 2007). Other portions of the mitochondrial genome have been sequenced in sea turtles, such as the cytochrome *b* coding region (Bowen et al. 1993) and ND4-Leucine coding region (Dutton et al. 1996); however, these regions are too conserved to be useful for population studies, and therefore additional sequence from the hypervariable control region show the most promise for detecting variation that may be informative for stock-structure analysis (Abreu-Grobois et al. 2006; Browne et al. 2010).

Improvements in sequencing technologies have allowed for larger mtDNA fragments to be easily and more accurately sequenced (Chan 2005). These improvements have enabled us to re-analyze specimens used in previous studies (Bass et al. 1996; Troëng et al. 2005) using new primers that provide longer sequences. The purpose of this study is to 1) determine whether additional genetic variation can be uncovered, 2) test if the additional variation can improve our capacity to detect population differentiation, and 3) use the new genetic survey to reanalyze the evolution of WC hawksbills. To do this, we carried out a comprehensive

stock structure re-assessment of WC hawksbill rookeries using longer sequence fragments, larger sample sizes, and additional rookeries that had not been included in previous surveys.

Methods

Sampling and Data Sets

Tissue specimens from nesting hawksbills have been collected as part of nesting site studies throughout the WC from 1993 to 2007 (Bass et al. 1996; Díaz-Fernández et al. 1999; Lagueux et al. 2003; Troëng et al. 2005; Lara-Ruiz et al. 2006; Vélez-Zuazo et al. 2008; Figure 1). Tissue types that were collected for DNA isolation include whole hatchlings, skin biopsies, and blood. Specimens are stored in 20% dimethyl sulfoxide (DMSO), saturated salt, or ethanol and are kept frozen at -20°C . Tissue samples from known females sampled during multiple nesting seasons were excluded to avoid sample replication. The present study is based on a compilation of 3 data sets: 1) existing specimens that were originally sequenced at 384 bp and 480 bp and were re-sequenced at the 832 bp length; 2) new specimens that have not been previously analyzed from Antigua (Jumby Bay), Costa Rica (Tortuguero), and the U.S. Virgin Islands (Buck Island), and new specimens collected from previously unstudied nesting sites in Nicaragua (Pearl Cays) and Guadeloupe (Marie Galante, Trois Ilets, and Galet Rouges, Basse-Terre); and 3) samples previously analyzed and published from Díaz-Fernández et al. 1999, Troëng et al. 2005, Lara-Ruiz et al. 2006, Vélez-Zuazo et al. 2008, and Browne et al. 2010 which were included to maximize sample size and geographic coverage if adequate sequence lengths were available (Figure 1; for further details, see, Supplementary Material).

Laboratory Analysis

For re-processing of tissue specimens (specimen sets 1 and 2), we isolated genomic DNA using a modified Qiagen DNEasy extraction kit, or by using standard manufacturer protocols with the X-tractor Gene robot (Corbett Robotics, San Francisco, CA, USA). We amplified approximately 832 bp of the the 5’ end of the mtDNA control region with polymerase chain reaction (PCR) using the primers LCM-15382 and H950g (Abreu-Grobois et al. 2006). Template DNA was amplified in a 25 μL PCR reaction using the profile described in Dutton et al. (2008). PCR products were purified and cycle sequenced in both directions using a 12 μL reaction consisting of a 1:1 buffered version of the ABI Big Dye Terminator v 3.1. Labeled extension products were purified using an ethanol-precipitation process and analyzed with an Applied Biosystems model 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). We aligned, analyzed, and edited sequences using Applied Biosystems SeqScape software v 2.5. Haplotypes were assigned by comparing aligned sequences against a reference library of master haplotypes. Variation beyond 740 bp was not detected. To compare

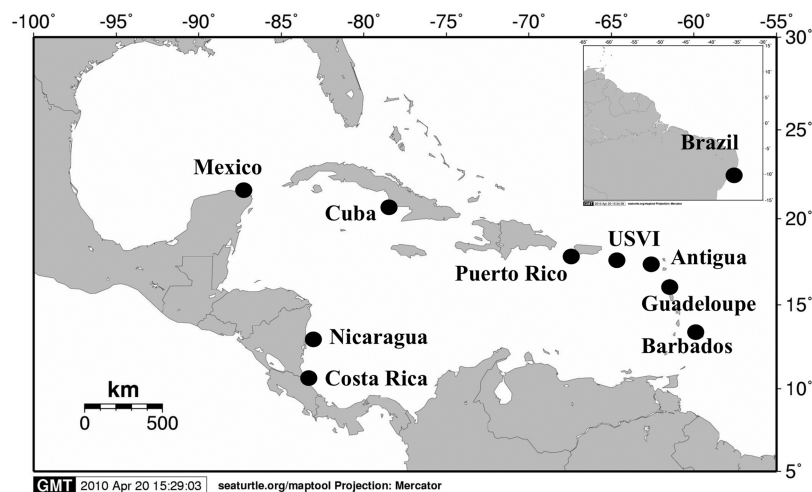


Figure 1. Sampling locations of hawksbill nesting populations in the Wider Caribbean.

with recent publications, 832bp sequences were trimmed to 740bp for further analyses.

Data Analyses

We calculated haplotype (h) and nucleotide (π) diversity for each rookery using Arlequin v 3.5.1.2 (Excoffier and Lischer 2010). We examined the distribution of polymorphism within the 740bp amplified sequences using the DnaSP program (Librado and Rozas 2009).

To find the most parsimonious phylogenetic relationships among haplotypes, we constructed a median-joining network with Network v 4.6 (Bandelt et al. 1999). To estimate haplotype abundances, we used relative haplotype frequencies from each rookery to convert to a proportion of each haplotype in the pooled data. Three ambiguity loops formed in the resulting network were resolved using the criteria of Pfenninger and Posada (2002). Phylogroups in the network were identified when a group of haplotypes was connected by a single haplotype in a monophyletic fashion with minimal differences.

We conducted further phylogenetic analyses to corroborate genetic differentiation among haplotype groups and to estimate the time to the most recent common ancestors (TMRCA) between the WC and selected hawksbill haplotype lineages from the East Atlantic (Príncipe Island; Monzón-Argüello et al. 2011), Indo-Pacific (Japan to Seychelles; Okayama et al. 1999), the Red Sea (Genbank acc. no. AJ421794.1), as well as outgroup species (*Caretta caretta*—Genbank acc. nos. EU179436.1 and EU179445.1; *Lepidochelys olivacea*—Genbank acc. no. U40661.1; *L. kempii*—Genbank acc. no. U40657.1). The best substitution model of sequence evolution (TrN+G, gamma shape = 0.017; Tamura and Nei 1993) was determined by jModelTest 0.1.1 (Posada 2008; Guindon and Gascuel 2003) and the Corrected Akaike Information Criterion and used in subsequent analyses. This we applied in the program BEAST v 1.6.1 (Drummond and Rambaut 2007; <http://beast.bio.ed.ac.uk>) using a relaxed

clock model (Drummond et al. 2006) and the subprogram BEAUti v 1.6.1 to prepare input files. In order to maximize the available sequence data, we used the 384bp data set, which is the most commonly available in published results. Following Naro-Maciel et al. (2008), we used times to most recent common ancestors based on fossil evidence and previous genetic studies as priors in the analyses with the following reference species splits: 1) between *Caretta* and *Lepidochelys* genera as 16 million years ago (mya) with a 95% confidence interval of 12–20 mya and 2) between *Lepidochelys olivacea* and *L. kempii* as 5 mya with a 95% confidence interval of 4–6 mya (Naro-Maciel et al. 2008). We examined posterior results with Tracer v 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>).

To determine the extent to which longer sequence data improved resolution of population structure in WC hawksbills, we compared and contrasted results of analyses using shorter (cropped to 384bp) versus longer sequence data. We performed pairwise F_{ST} (conventional frequency based) and Φ_{ST} (Tamura-Nei, gamma = 0.017) comparisons, exact tests of population differentiation, and analysis of molecular variance (AMOVA) using Arlequin. No Bonferroni corrections for multiple tests were applied (Perneger, 1998; Nakagawa 2004).

We used the program POWSIM (v 4.0; Ryman and Palm 2006), adjusted for organelle (mtDNA) data (Larsson et al. 2009) to evaluate the statistical power of the shorter and longer sequences to detect genetic differentiation at various levels of F_{ST} . We simulated population drift to F_{ST} levels of 0.005, 0.01, and 0.05 by using an effective population size (N_e) of 1000 and varying the number of generations (t) accordingly (Ryman et al. 2006). We determined statistical power for two levels of variation corresponding to the 12 haplotypes from the shorter (384bp) sequences and 23 haplotypes from the longer (740bp) sequences, 11 populations and sample sizes of 20, 50, and 100. Power is expressed as the proportion of significant outcomes (1000 replicates, rejecting the null hypothesis (H_0) of no allele frequency

difference, or $F_{ST} = 0$ at $P < 0.05$) providing estimates of the probability of false negatives for population differentiation at the expected degree of divergence (Ryman et al. 2006).

We constructed extended majority-rule consensus dendrograms for pairwise rookery F_{ST} values for 384 and 740 bp results under the neighbor-joining model and incorporated bootstrap data using MEGA v 4.1 (Tamura et al. 2007). We used results from 1000 F_{ST} distance matrices generated from bootstrap re-samplings of the rookery haplotype profiles with Excel macros as input for Arlequin under the batch mode. We generated the corresponding number of neighbor-joining trees with the Neighbor module, and identified the consensus tree with the Consense module with algorithms provided in the computer package Phylip v 3.67 (Felsenstein 2005).

Spatial analysis of molecular variance or SAMOVA (Dupanloup et al. 2002) was used to identify groups of populations whose geographic distance is minimized while maximizing differentiation from each other. We also tested isolation by distance models through the use of Mantel tests (Mantel 1967) by evaluating the relationships between geographic distances and the corresponding rookery pairwise values of 1) genetic distances (F_{ST}) and 2) Euclidean distances using clade frequencies. Distances (km) between rookeries were obtained from estimates using Google Earth and the shortest sea routes not going over land. For the F_{ST} Mantel test, geographic distances were transformed using natural logs, while frequency-based F_{ST} values were standardized using $F_{ST}/(1-F_{ST})$ and run in Arlequin using 10 000 permutations. The Mantel test with clade frequencies were carried out in XLSTAT v 2011.3.02 (www.xlstat.com) on untransformed data and with Pearson's correlation coefficients.

Arlequin was used to assess for fit of demographic (Rogers and Harpending 1992) and geographic (Ray et al. 2003) expansion or contraction through a mismatch-distribution analysis using only the longer sequence information from (a) individual rookeries, (b) pooled data for the entire WC, and (c) data pooled by clade. Parameters for the sudden demographic expansion estimated included tau (τ) and the θ -estimates $\theta_0 = 2N_0\mu$, and $\theta_1 = 2N_1\mu$, where μ is the mutation rate and N_0 and N_1 are the female effective population sizes before (time 0) and after (time 1) expansion. For the geographic expansion model, τ , as well as $\theta = \theta_0 = \theta_1$ and $M = 2N\mu$ (θ is $2N\mu$ at demographic equilibrium and m is the rate at which the sampled deme would exchange migrants with a unique population of infinite size after T generations) were estimated. Time in generations since expansion was estimated as $T = \tau/2\mu$ (where μ is the mutation rate). Goodness of fit was assessed by the raggedness index (Rogers and Harpending 1992) and the sum of square deviations (SSD) between the observed and expected mismatch by parametric bootstraps. Tajima's D test (Tajima 1989) and Fu's F_s statistic (Fu 1997) also implemented in Arlequin were used to test for departures from neutrality due to recent population expansions or selection. Effective female population sizes (N_{ef}) were calculated from the formula $\theta = 2N_{ef}\mu$ (Tajima 1993), where μ is the mutation rate per sequence per generation. To solve equations involving μ and generation times, we used

a mutation rate of 2% (range: 1.2%–2.4%) sequence divergence per million years between pairs of lineages (Dutton et al. 1996; Encalada et al. 1996) and a generation time of 35 years (Mortimer and Donnelly 2008). The mutation rate per lineage we used was therefore 0.6–1.2% per million years.

Results

Analysis of Haplotype and Nucleotide Diversity

Based on 647 specimens from 10 nesting populations we examined sequence data that span the majority of the mitochondrial control region including the 384 bp and 480 bp segments previously reported (Table 1). A microsatellite region at the 3' end that reportedly manifests heteroplasmy in at least some marine turtle species was avoided (Laurent et al. 1998). We also incorporated previous results from an 11th rookery in Cuba ($n = 70$) sequenced at 480 bp (Díaz-Fernández et al. 1999).

We identified 21 polymorphic sites that describe 23 haplotypes including those described in the original publication for the 480 bp Cuban sequences (Díaz-Fernández et al. 1999). Nineteen of the variable sites were transitions and one was a transversion. In addition, we identified a 10 bp insertion (Table 1). We detected additional polymorphic sites with the longer reading frame at both the 5' and 3' ends of the control region outside of the original 384 bp segment (Figure 2). Nonetheless, we found that about 75% of the total site variability of the WC control region remained within the 384 bp segment that was previously studied.

It was possible to further differentiate 5 of the original haplotypes reported by Bass et al. (1996) into 11 haplotypes based on new variation detected with the additional sequence data (Table 2). Seven of these were previously reported with 480 bp readings (Díaz-Fernández et al. 1999), but the remaining 4 haplotypes are a result of sequencing beyond 480 bp. In addition, 3 new haplotypes were identified based on variation within the 480 bp region (EiA62–Lara-Ruiz et al. 2006, EiA65 and EiA84). Although it is possible that longer sequences might reveal new variation in the Cuban specimens, it is noteworthy that with the exception of EiA1 and EiA30 the remaining Cuban haplotypes are either exclusive to this population (EiA13, EiA29) or the additional variants with respect to the 384 bp sequences have already been detected within the first 480 bp (EiA11; Table 2).

Haplotype diversities within nesting sites based on the 740 bp sequences ranged from a lowest value of $h = 0.000$ (for Barbados-Leeward where haplotype EiA1 was fixed) to 0.655 (at Costa Rica). Nucleotide diversities ranged from $\pi = 0.000$ (Barbados-Leeward) to 0.0240 (Costa Rica; Table 3; see below). As expected, h values tended to be higher with 740 bp sequences as a result of increased numbers of haplotypes being detected. However, the π values (with the exception of the Holbox, Mexico rookery hereafter referred to as "Mexico") were higher from the 384 bp readings (Table 3) due to the fact that the density of variation (variability/site) actually decreases before and after the 384 bp reading frame (see Figure 2).

Table 1 Mitochondrial DNA control region polymorphisms and haplotype designations for Wider Caribbean hawksbill specimens assayed with 832 bp sequences, indicating corresponding 384 bp and 480 bp haplotypes previously published by Bass et al. (1996) and Diaz-Fernández et al. (1999), respectively

Nomenclature	Diaz-Fernández et al. 1999	Pos from start of Dloop / New Nomenclature	Diaz-Fernández et al. 1999 reading frame																												Genbank accession number
			Bass et al. 1996 reading frame														Diaz-Fernández et al. 1999 reading frame														
			85	109	123	157	170	186	204	213	266	318	360	363	365	417	418	449	452	500	576	649	662	662							
A	Cu1	EiA1	C	A	A	A	T	A	A	A	G	C	T	A	T	A	T	G	G	—	G	T	G	C	A	JN998509					
Alpha	g	EiA61*																								DQ177339.1					
B	e	EiA2	T	G	G	G						C	C													JN998513					
		EiA52										C	C													JN998524					
F	c	EiA3	T	G	G	G	C	T	C	G	G	T	C	A	C	A	A	—	C	C		C	C			JN998519					
		EiA9	T	G	G	G	C	T	C	G	G	T	C	A	C	A	A	—	C	C		C	C			JN998526					
F with 10bp insert	PR1	EiA11	T	G	G	G	C	T	C	G	G	T	C	A	C	A	A	—	C	C		C	C			JN998510					
G	PR1 with 10bp insert	EiA84	T	G	G	G	C	T	C	G	G	T	C	A	C	A	10bp insert	—	C	C		C	C			JX441892					
Gamma	i	EiA12	A																JN998511					
L	Cu2	EiA13	T	G	G	G	C	C	T	C	G	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998512					
	PR3	EiA18	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998523					
N	PR2	EiA47	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998514					
O	PR4	EiA20	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998515					
P	MX3	EiA21													G											JN998516					
	MX1	EiA22	T	G	G	G	C	G	T	C	C	T	C	C	C	A	—	—	—	—	—	—	—	—	—	JN998521					
Q		EiA23	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998517					
	MX2	EiA41	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998522					
	Cu3	EiA43	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998518					
	Cu4	EiA29	T	G	G	G	C	C	T	C	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	JN998520					
	f	EiA30	T	G	G	G	C	G	G	T	C	T	C	C	A	A	—	—	—	—	—	—	—	—	—	DQ177340.1					
		EiA32*					G																			JN998525					
		EiA65	T	G	G	G	C	C	T	C	G	T	C	C	A	A	—	—	—	—	—	—	—	—	—	DQ177341.1					
		EiA62*													G											JN998525					

"?" refers to missing data in haplotypes that have not been sequenced at the longer length. Haplotype sequences (740 bp) published by Lara-Ruiz et al. (2006) are indicated by an asterisk.

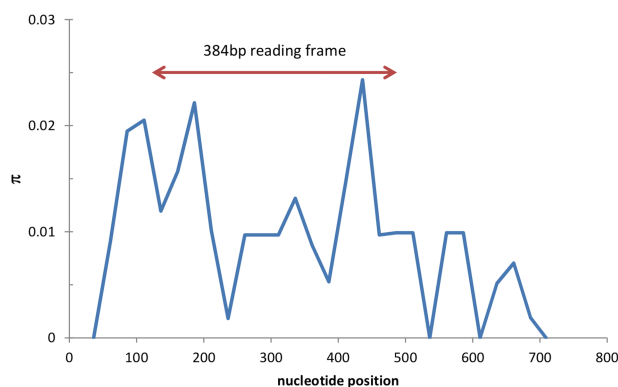


Figure 2. Population-wide distribution of nucleotide diversity (π) in the assayed 740 bp segment of the mtDNA control region demonstrating the presence of polymorphic sites extending outside the 384 bp segment traditionally used. The distribution of the variation along the set of sequences was estimated using the sliding window option, with a window length of 50 sites and a step size of 25 sites.

The most parsimonious median-joining network of haplotype sequences indicated two broadly divergent phylogroups separated by 6 substitutions (including the single transversion found) between haplotypes clustering around Eia1 in the first, and around Eia23 and Eia11 in the second (Figure 3). Central haplotype Eia1 (one of the two most abundant haplotypes) defined clade 1 which exhibited a classic star shape, and 7 closely related haplotypes differentiated by a single substitution each clustered around it. The second most abundant haplotype Eia11, also a central haplotype, formed clade 2 with 6 haplotypes removed by 1 substitution and 4 others with greater differentiation. Clade 3 formed around central haplotype (Eia23) mostly observed in Mexico at appreciable frequencies, and haplotype Eia29 only from Cuba at low frequency.

While haplotypes from clade 3 were mostly concentrated in Mexico, clade 1 and 2 haplotypes were widely distributed in the region. Nevertheless, haplotypes from clade 2 appeared to be clustered at relatively high frequencies (> 0.40) in the eastern and western Caribbean whereas clade 1 haplotypes were found at high frequencies in widely dispersed sites (Cuba, Antigua, and Brazil). It was notable that Brazil not only had haplotypes only from clade 1 but out of a total of 7 haplotypes observed in this study from this clade, 4 were found there. The relationship between geographic distribution and the rookery composition by clades is analyzed below.

Our broader phylogenetic analysis involving sequence data for Atlantic and Indo-Pacific regions revealed a deep split between WC and eastern Atlantic-Indo-Pacific mtDNA lineages with a divergence date from Bayesian estimates of about 4.5 mya (95% highest posterior density interval (HPDI): 1.2–8.2 mya; Figure 4). The analysis also produced estimates of the time to most recent common ancestor (TMRCA) for the 3 clades identified in our network for the WC hawksbills.

Phylogroup I (with a single clade) appeared to have split earliest from the rest of the WC lineages, with a TMRCA with phylogroup II estimated at 2.2 mya (95% HPDI: 0.5–4.7 mya). Separation between clades 2 and 3 in the latter phylogroup appeared to have occurred much more recently, with a TMRCA of about 1.2 mya; 95% HPDI: 0.3–2.3 mya).

Interpopulation Analysis

Results of the AMOVA analyses were very similar for the 384 bp and the 740 bp data, and corroborate significant structuring among populations ($F_{ST} = 0.508$ and 0.504 ; $\Phi_{ST} = 0.539$ and 0.548 , respectively for 384 bp and 740 bp, all $P < 0.0001$, results not shown). The number of significant pairwise rookery comparisons increased for all 3 measures we examined (F_{ST} , Φ_{ST} , and the exact test of population differentiation) when the longer sequence data were used. When using the 740 bp data, only a single comparison (Barbados-Windward vs. USVI) was nonsignificant in the F_{ST} pairwise comparisons. Conversely, all the F_{ST} , Φ_{ST} , and the exact test comparisons involving Guadeloupe became significant with 740 bp (Table 4a, b).

Our POWSIM simulations showed that both the shorter and longer sequence markers have probabilities of $> 90\%$ for detecting an F_{ST} value ≥ 0.01 when there are 11 populations and where sample sizes > 20 . Power for detecting weak differentiation ($F_{ST} = 0.005, 0.01$) was low for both markers at low sample size ($N = 20$), however, power was consistently greater when the number of haplotypes was increased at each sampling level, and when $N < 30$ increasing the number of haplotypes was as effective in increasing power as increasing sample size. We found a 100% probability of detecting population differentiation of $F_{ST} \geq 0.05$ with both markers at all sampling levels (Supplementary Figure 1a and b).

The population NJ consensus trees depicting the phylogenetic relationships using F_{ST} values resolved 3 major branches with the 384 bp data (Figure 5a) and a fourth branch distinguishing Guadeloupe with the longer 740 bp sequences (Figure 5b). The major population phylogenetic branches revealed in this tree contained 1) rookeries characterized by haplotype profiles dominated by Eia1 (Antigua, Brazil, Barbados-L, and Cuba); 2) rookeries with major haplotype being Eia11 (Barbados-W, Costa Rica, Nicaragua, Puerto Rico and USVI); 3) Mexico with a highly divergent haplotype profile and extremely small haplotype overlap with other populations; and (4) Guadeloupe, characterized by the newly revealed haplotype profile with a unique predominance of the Eia9 haplotype and low frequencies of haplotypes shared with other rookeries.

Our SAMOVA results indicated that maximal differentiation between groups for the set of rookeries under study was obtained with five groups ($F_{CT} = 0.532$; $P < 0.001$) where the grouping was as follows: 1) Puerto Rico, Nicaragua, Costa Rica, USVI, Barbados-Windward; 2) Mexico; 3) Barbados-Leeward, Cuba, Brazil nonhybrids; 4) Antigua; and 5) Guadeloupe.

Results of the Mantel tests comparing geographic distance with F_{ST} values for intrapopulation proportion of the

Table 2 Haplotype profiles for 11 Wider Caribbean rookeries with information on haplotype frequency and sample sizes. The new nomenclature represents current haplotype names

Bass et al. 1996 nomenclature	Díaz-Fernández et al. 1999 nomenclature											New												
	A	Alpha	B	F	G	gamma	L	N	O	P	Q													
	Cu1	g	e	c	PR1	i	Cu2	PR3	PR	PR4	MX3	MX1	MX2	Cu3	Cu4	f								
Nomenclature	EIA1	EIA61	EIA2	EIA52	EiA3	EiA84	EiA12	EiA13	EiA18	EiA47	EiA20	EiA21	EiA22	EiA23	EiA41	EiA43	EiA29	EiA30	EiA32	EiA62	EiA65	N		
Antigua	42		29		1																		72	
Barbados	54																						54	
– Leeward*																								
Barbados	3			6	21																		30	
– Windward*																								
Brazil	52	4																	9	1			66	
nonhybrids**																								
Costa Rica		11	1	3	33	5			6														60	
Cuba***	62				1		5										1						70	
Guadeloupe -	2			69		1																	72	
Trois Îlets, Marie																								
Galante																							2	
Guadeloupe -																								
Galet Rouges,																								
Basse-Terre																								
Mexico													2	16	1	1							20	
Nicaragua		19			54	5																	20	
Puerto Rico****	3			2	60		1				34	6										1	95	
USVI	8		2	2	50						4			1									109	
Total per	226	4	30	1	31	82	222	5	6	5	1	6	38	6	2	17	1	20	1	2	9	1	1	717
Haplotype																								

*from Browne et al. 2009, **from Lara-Ruiz et al. 2006, ***from Díaz-Fernández et al. 1999, ****from Velez-Zuazo et al. 2008. In the analyses for this study, Barbados-Leeward and Barbados-Windward were included as separate rookeries, whereas, Guadeloupe—Trois Îlets, Marie Galante and Guadeloupe—Galet Rouges, Basse-Terre were grouped into a single population

Table 3 Haplotype and nucleotide diversities for rookeries calculated using 384bp and 740bp of the mtDNA control region sequences.

	Current census size (nests/yr)	Ref	384bp						740bp				
			No. of Haplotypes	h	s.e. h	π	s.e. π	No. of Haplotypes	h	s.e. h	π	s.e. π	
Antigua	203	1	72	3	0.504	0.027	0.0289	0.0147	3	0.504	0.027	0.0179	0.0090
Cuba_DL	130	1	70	5	0.213	0.064	0.0186	0.0098	5	0.213	0.064	0.0167	0.0087
Barbados L	1504	1	54	1	0.000	0.000	0.0000	0.0000	1	0.000	0.000	0.0000	0.0000
Brasil_nH	304	2	66	3	0.265	0.064	0.0011	0.0011	4	0.362	0.069	0.0007	0.0006
Costa Rica	25	1	60	5	0.593	0.059	0.0644	0.0318	7	0.655	0.057	0.0240	0.0120
Nicaragua	205	5	95	5	0.612	0.042	0.0510	0.0252	5	0.612	0.042	0.0186	0.0094
Barbados W	150	6	30	2	0.186	0.088	0.0167	0.0091	3	0.476	0.091	0.0086	0.0047
Puerto Rico	740	1	109	6	0.580	0.036	0.0228	0.0117	7	0.600	0.035	0.0098	0.0051
USVI	158	1	67	5	0.384	0.071	0.0201	0.0105	6	0.430	0.072	0.0101	0.0053
Guadeloupe	151	3	74	3	0.080	0.043	0.0080	0.0047	4	0.131	0.053	0.0033	0.0020
Mexico_Hbx	311	4	20	2	0.190	0.108	0.0007	0.0009	4	0.363	0.131	0.0015	0.0011
pooled data	3881		717	17	0.709	0.012	0.0492	0.0241	23	0.785	0.010	0.0227	0.0112

Current census sizes (nests/yr) were obtained from the following: 1. Velez-Zuazo et al. 2008; 2. Lara-Ruiz et al., 2006; 3. Kamel and Delcroix 2009; 4. Cuevas et al. 2007; 5. Lagueux et al. 2003; Lagueux and Campbell, unpublished data; 6. J. Horrocks, unpublished data.

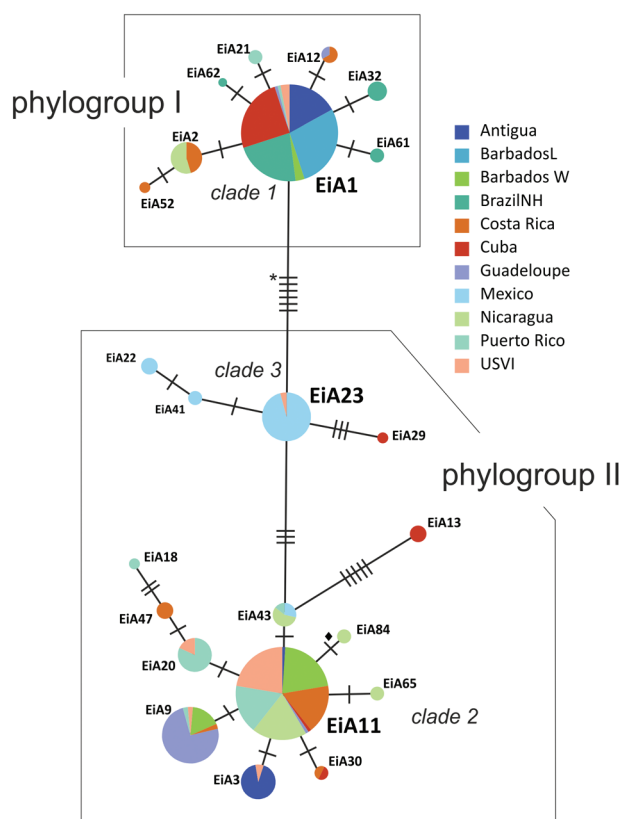


Figure 3. Most parsimonious median-joining network of the 740bp Caribbean hawksbill haplotypes. Number of mutations between haplotypes is illustrated by dashes in connecting lines and correspond to data in Table 2. The position of the single transversion (asterisk) and a 10bp insertion (solid diamond) are indicated. The size of the circles is approximately proportional to haplotype frequency in the overall sample set. Colors denote the locations where individual haplotypes were detected and the proportions of shared haplotypes that were distributed among different rookeries. See Material and Methods for definition of phylogroups and clades.

3 clades were nonsignificant ($R^2 = 0.000011$, $P = 0.972$ and $R^2 = 0.0301$, $P = 0.847$; F_{ST} and Slatkin's (1995) linearized F_{ST} respectively) indicating that a simple model of isolation by distance does not apply. Nevertheless, a Mantel test for a comparison between proportions of clades in individual populations against the interpopulational geographic distances was significant ($R^2 = 0.076$; $P = 0.040$; Supplementary Figure 2). This latter result may be the consequence of the clustering of clade 2 at high frequencies among rookeries in the eastern Caribbean and in central America.

Mismatch Analyses and Effective Population Size

Tracing of historical demography of populations using mismatch analysis assumes mutation-drift equilibrium (Slatkin and Hudson, 1991; Rogers and Harpending 1992). Hence, when there is secondary contact, it is appropriate to carry out the historical demography analysis separately for each clade to avoid Type II errors (Alvarado-Bremer et al. 2005). Given that our preliminary results indicated the presence of more than one clade and strongly bimodal distributions in mismatch analyses, indicating secondary contact, we used molecular data for the 3 EC clades (instead of individual rookeries) and data pooled for the entire region to allow comparison with previous reports.

Significant departure from neutrality (indicating population size expansion) was observed for clades 1 and 3 but not for clade 2. In addition, and congruent with an interpretation of demographic expansion from the results, the mismatch data for clades 1 and 3 fit a model of demographic expansion, whereas only clade 3 fits a model of spatial expansion as well (P values for SSD and RG index > 0.05 ; Supplementary Table 1). In contrast, the pooled WC data was found to fit neutrality, yet the data fit a demographic expansion model in spite of a strong bimodal distribution of the mismatch analyses (not shown).

Mismatch analyses indicates $\tau = 0.99$ – 3.0 for the 3 clades which, when combined with the θ results, correspond to bottlenecks occurring between about 100 000 to 300 000 years ago (Table 5). Estimates for the current effective female population size (N_{ef}) derived from the clade data but using

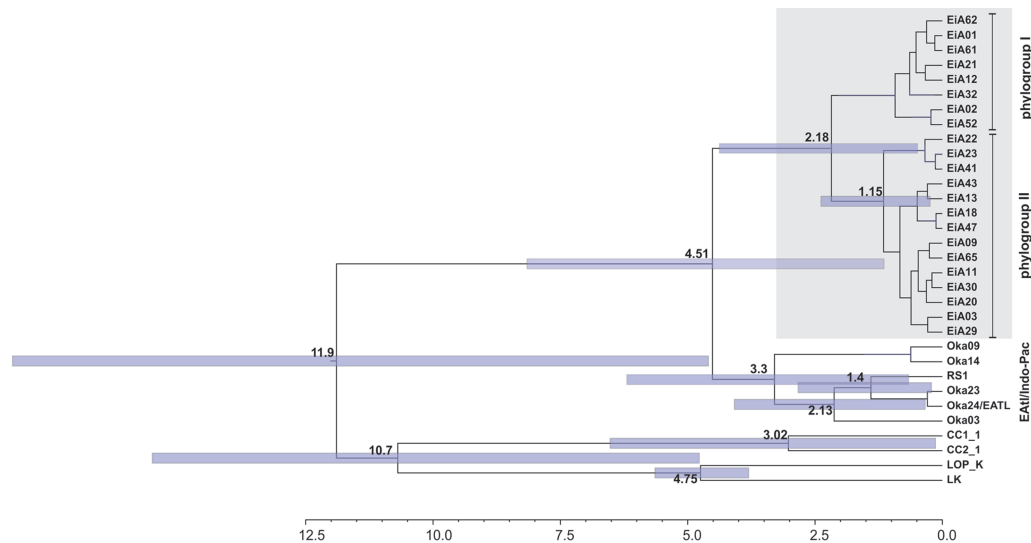


Figure 4. Divergence times (million years before present, x-axis) calculated using the program BEAST for the coalescents among 384 bp hawkbill mtDNA control region lineages from the Wider Caribbean and selected regions. Mean highest posterior density (HPD) values estimated for tree nodes are indicated together with their corresponding 95% HPD intervals (horizontal bars). Sources for regional data sets: East Atlantic (EATL; [Monzón-Argüello et al., 2011](#)), Indo-Pacific (denoted by “Oka” prefix and correspond to haplotypes from Japan to Seychelles described in [Okayama et al., 1999](#)), and the Red Sea (Genbank acc. no. AJ421794.1). Loggerhead (haplotypes 1.1 and 2.1 Genbank acc. nos. EU179436.1 and EU179445.1), Pacific olive ridley (LOP_K; Genbank acc. no. U40661.1) and Kemp’s ridley (LK; Genbank acc. no. U40657.1) sequences were included as outgroups and to incorporate time calibration from fossil evidence (see text). Shaded box shows the Wider Caribbean hawkbill lineages from our study, phylogroups correspond to those identified from [Figure 3](#).

the θ values per sequence indicate N_{ef} values of between 1012 and 3977 for individual clades. Summing these N_{ef} values results in a N_{ef} value of between 6000 and 9000 for the entire WC region. N_{ef} values derived from the pooled WC data resulted in an estimate ten times larger (36 000–54 000; [Table 5](#)).

Discussion

Our results illustrate that longer sequences are more informative for describing the mtDNA variation among hawkbill populations than the shorter sequences used in previous studies. The primers used in this study extend the sequencing reading frame into additional polymorphic portions of the mtDNA control region. This allows for the identification of more variation within hawksbills, thus yielding better resolution in hawkbill stock structure among some WC rookeries and meeting a critical need for hawkbill management objectives in the region.

However, any improved capacity to discriminate otherwise genetically indistinguishable populations will only occur when changes in haplotype frequencies, among otherwise undifferentiated populations, are both large and contrasting between the populations. The most notable improvement to distinguish populations occurred when longer sequences were used to assay Guadeloupe as a distinct demographic unit. Another major improvement was the identification of 3 haplotype variants of the previous Q haplotype: EIA23, EIA41, and EIA43. Within Mexico where these 3 haplotypes

are widespread, differences in their relative frequencies will allow further distinction of population units (Abreu, unpublished data). These relationships would not be detected with the shorter sequences.

Almost all pairwise comparisons involving Guadeloupe changed substantially when using the longer as compared to the shorter sequences, while comparisons involving the other rookeries changed very little. The reason for this is primarily due to the degree of overlap in haplotype frequencies in the pairwise comparisons. In cases where different populations share a relatively high number of the shorter (384 bp) haplotypes and these haplotypes subsequently are split into new variants using the longer (740 bp) sequences, the resulting change can be substantial.

One primary conservation concern is the incorrect grouping of apparently genetically indistinguishable populations when in fact they are reproductively isolated. Thus, a much better indicator of increased stock resolution afforded by the longer sequences would be the proportion of nonsignificant pairwise F_{ST} values. Based on the 384 bp sequences, two out of the 55 comparisons were nonsignificant ([Table 4](#); USVI vs. Barbados-W and Guadeloupe vs. Barbados-W) while with the longer sequences, although still indicating the former comparison as nonsignificant, identified Guadeloupe as a genetically distinct population ($F_{ST} = 0.683$, $P < 0.05$). A major advantage results if widespread and high-frequency haplotypes are split and generate large frequency shifts.

Our study builds on previous hawkbill stock structure surveys by filling in major gaps in geographic coverage in

Table 4 Population genetic subdivision among Wider Caribbean hawksbill rookeries. (A) Pairwise F_{ST} using 384bp data (below diagonal) and 740bp data (above diagonal) and (B) Φ_{ST} using 384bp data (below diagonal) and 740bp data (above diagonal)

	Antigua		Cuba		Barbados-Leeward		Brazil nonhybrids		Costa Rica		Nicaragua		Barbados-Windward		Puerto Rico		USVI		Guadeloupe		Mexico	
(A)																						
Antigua																						
Cuba	0.257																					
Barbados-Leeward	0.257	0.257																				
Brazil nonhybrids	0.361	0.058	0.197																			
Costa Rica	0.235	0.038	0.132	0.419																		
Nicaragua	0.449	0.603	0.576	0.573	0.434																	
Barbados-Windward	0.466	0.598	0.576	0.661	0.501	0.471																
Puerto Rico	0.585	0.773	0.741	0.495	0.027	0.107	0.142															
USVI	0.441	0.566	0.545	0.495	0.027	0.107	0.113	0.164														
Guadeloupe	0.509	0.663	0.638	0.087	0.100	0.096	0.100	0.007	0.087													
Mexico	0.701	0.850	0.828	0.221	0.222	0.245	0.222	0.017	0.083	0.528	0.528	0.439	0.528	0.528	0.450	0.450	0.556	0.556	0.679	0.679	0.543	
(B)																						
Antigua																						
Cuba	0.157																					
Barbados-Leeward	0.374	0.083	0.417	0.167	0.268	0.439	0.268	0.439	0.450	0.528	0.528	0.439	0.528	0.450	0.450	0.556	0.556	0.679	0.679	0.825	0.825	
Brazil nonhybrids	0.437	0.138	0.056	0.423	0.514	0.674	0.514	0.674	0.674	0.722	0.722	0.674	0.722	0.674	0.759	0.759	0.825	0.825	0.923	0.923	0.744	
Costa Rica	0.097	0.367	0.549	0.619	0.683	0.921	0.683	0.921	0.860	0.858	0.858	0.920	0.858	0.860	0.949	0.949	0.825	0.825	0.753	0.753	0.896	
Nicaragua	0.192	0.455	0.607	0.650	0.705	0.921	0.705	0.921	0.869	0.868	0.868	0.921	0.868	0.869	0.946	0.946	0.825	0.825	0.599	0.599	0.637	
Barbados-Windward	0.529	0.759	0.929	−0.009	0.000	0.074	0.000	0.074	0.082	0.159	0.159	0.074	0.159	0.082	0.184	0.184	0.825	0.825	0.636	0.636	0.505	
Puerto Rico	0.575	0.756	0.859	0.094	0.042	0.094	0.042	0.094	0.002	0.084	0.084	0.030	0.084	−0.019	0.151	0.151	0.825	0.825	0.683	0.683	0.573	
USVI	0.522	0.738	0.876	0.103	0.040	−0.030	0.040	−0.030	0.006	0.002	0.002	−0.030	0.002	0.006	0.115	0.115	0.825	0.825	0.594	0.594	0.476	
Guadeloupe	0.656	0.833	0.948	0.212	0.128	−0.023	0.128	−0.023	0.023	0.245	0.245	0.017	0.245	0.083	0.710	0.710	0.825	0.825	0.710	0.710	0.587	
Mexico	0.583	0.797	0.987	0.116	0.103	0.209	0.103	0.209	0.151	0.528	0.528	0.812	0.528	0.671	0.895	0.895	0.825	0.825	0.895	0.895	0.809	

See Material and Methods for data set sources. All results with P -values < 0.05 are in bold type.

Table 5 Time since population expansion inferred from mismatch analyses, current and historical N_{ef} sizes estimated for Caribbean hawksbills

	Pooled WC data	Clade 1	Clade 2	Clade 3	Combined clades	
Historical inferences						
Theta initial per sequence (θ_0)	0.002	0.000	0.004	0.000		
Tau (τ)	14.838	3.000	0.992	3.000		
Max time since population expansion $t = \tau/2u$ [Generations]	47 741	9653	3192	9653	7499	(avg)
Min time since population expansion $t = \tau/2u$ [Generations]	31 827	6435	2128	6435	4999	(avg)
Max time since expansion [years]	1 670 934	337 838	111 712	337 838	262 462	(avg)
Min time since expansion [years]	1 113 956	225 225	74 474	225 225	174 975	(avg)
N_{ef} prior to population expansion:						
Max $N_{ef} = \theta_0 / (2 \times u)$	6	0	11	0	11	(sum)
Min $N_{ef} = \theta_0 / (2 \times u)$	4	0	8	0	8	(sum)
Current estimates						
$\theta\pi$ per sequence	16.84	0.47	1.24	1.06	2.76	(sum)
Max current $N_{ef} = \theta / (2 \times u)$	54 180	1518	3977	3401	8896	(sum)
Min current $N_{ef} = \theta / (2 \times u)$	36 120	1012	2652	2267	5931	(sum)

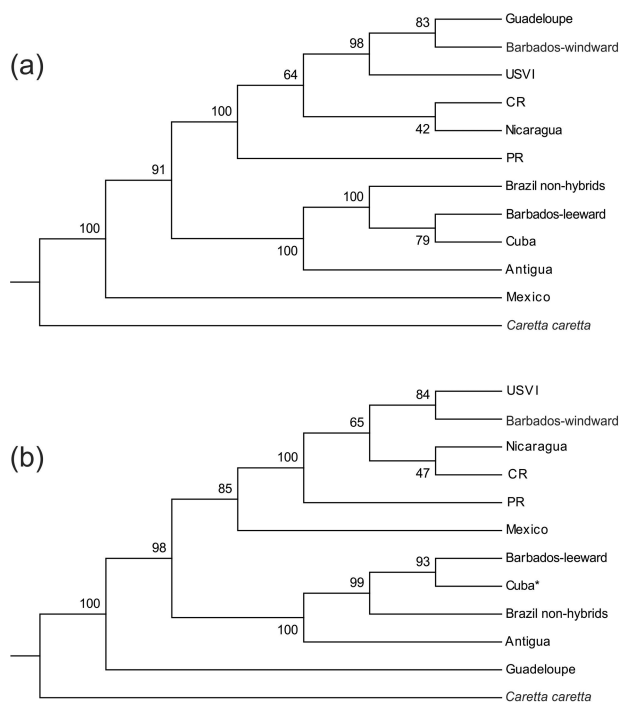


Figure 5. Neighbor-joining tree of the genetic relationships (conventional pairwise F_{ST} values) among Wider Caribbean hawksbill rookeries based on (a) 384 bp of control region sequence and (b) 740 bp of control region sequence. Bootstrap values from 1000 replicates are shown on the corresponding nodes. Loggerhead turtle (*Caretta caretta*) sequence CC-A1 (GenBank accession number AJ001074) was used as outgroup. *Denotes that data for Cuba was only available at the 480 bp length.

the WC, and also resolves some enigmatic results from earlier work. For instance, the re-analysis of the original specimens from Barbados indicated that haplotypes D and

E originally reported by Bass et al. (1996) appear to errors resulting from older sequencing technology (Browne et al. 2010). These haplotypes (D & E) along with haplotype C, originally found in Antigua (Bass et al. 1996); D11 in Mexico (Bass et al. 1999); and J, K, & M in Puerto Rico (Bass et al. 1996), have not been detected among any of the nesting or foraging populations subsequently studied. The absence of the Puerto Rican haplotypes previously reported (J, K, & M) also helps resolve questions raised by recent studies regarding the temporal shifts in haplotype composition of this nesting stock (Vélez-Zuazo et al. 2008). In some extreme cases, the modifications resulting from the new haplotype profiling may significantly alter the outcome of mixed stock contribution estimates for foraging aggregations (see Blumenthal et al 2009; Browne et al 2010).

We did not find any variation in the last 170 bp from the longer 832 bp read. We also included the Cuban data published by Díaz-Fernández et al. (1999) in our stock-structure analysis although the haplotypes were based on 480 bp sequences. Because the variants of the F haplotype group are defined by mutations occurring in the first 110 bp, which is within the Díaz-Fernández et al. (1999) reading frame, and the majority (62 out of 70 individuals) correspond to EiA1, which is not further differentiated in any of the specimens we sequenced beyond the Díaz-Fernández et al. (1999) reading frame, we felt it was reasonable to include this data set in our analysis for heuristic purposes. The other two haplotypes observed in Cuba (EiA13 and EiA29) were detected only in Cuba, while haplotype EiA30 has also been found in one Costa Rica specimen. It is possible however that re-analysis of the Cuban specimens with the new primers may yield new variants of EiA1 as a result of mutations that might be detected outside the Díaz-Fernández et al. (1999) reading frame. Overall, given that we did not detect any variation before bp 82 and beyond bp 662, we conclude that it is reasonable to edit sequences to the 740 bp fragment to designate haplotypes as it is unlikely that the current results

on interpopulation differentiation would be modified if novel variation were found in the Cuban rookery. This is the fragment generated by the LTEi9 and H950 primers (Abreu-Grobois et al. 2006), however, we recommend using the primers LCM-15382 and H950 to generate the 832 bp fragment and cropping the sequence to 740 bp for consistency and to obtain cleaner reads at the ends.

Our POWSIM results indicate that both longer and shorter sequences have high probability of detecting the levels of population differentiation found among many of the WC hawksbill populations in our study, and that when F_{ST} was greater than 0.05, sampling as few as 20 individuals per population should be sufficient for either markers. Increasing the sequence length, and therefore the number of haplotypes does not require larger sample sizes to maintain sufficient statistical power to detect differentiation; in fact, power was consistently improved by increasing the number of haplotypes (Figure 5). However, for weak population differentiation ($F_{ST} \leq 0.01$), increasing sample sizes to 50 or more will improve power more than increasing the number of haplotypes. This is consistent with findings reported by Kalinowski (2005), who also concluded that larger sample sizes were not required to detect population differentiation with polymorphic nuclear markers, unless F_{ST} was less than 0.01. Variability of the loci seems to be a more important influence on power to detect population differentiation than the sample size, as long as the lowest threshold of sampled individuals has been reached (Björklund and Bergeck 2009).

Additional support for the pattern of genetic differentiation we see in the F_{ST} analysis comes from the results of our SAMOVA analysis. The optimal grouping detected by SAMOVA broadly coincides with the topology of the NJ consensus trees based on F_{ST} values, with the exception that better group differentiation was obtained with Antigua as a separate entity. This is consistent with the consensus tree, which has Antigua as a distal node to the Brazil-Cuba-Barbados cluster, as the Antigua rookery does contain EiA3 at a distinctly high frequency (F_{ST} , F_{SC} , and F_{CT} all < 0.0001). Bowen et al. (2007b) suggest that the pattern of differentiation found for hawksbills rookeries indicated a western versus eastern Caribbean division separated by the high-energy Caribbean current. Blumenthal et al. (2009) incorporated ocean drift models and concluded that the complexities of local and regional current patterns likely drive geographic patterns of genetic diversity in hawksbills in the WC which could help to explain the patchy relationships suggested by our data.

Demographic History and Phylogeography

Overall, our findings do not refute any of the broader conclusions made on stock structure based on the shorter sequences used in previous studies (Bass et al. 1996; Bowen et al. 2007b). However, our additional data from the new rookeries, combined with the increase in information from the longer sequences introduces a more complex phylogeographic paradigm that does not fit an isolation by distance model prevalent in the published literature (Wright 1943; Reece et al. 2005;

Reis et al. 2010). Instead, it reflects a curious mixed pattern where relatedness is patchy and we find connectivity between geographically distant rookeries while only a few proximate rookeries are genetically similar (Figure 6), suggesting episodic dispersal. This is also reflected in the haplotype network (Figure 3), which shows that, with the exception of Brazil and Mexico, all other rookeries contain haplotypes from more than one clade. Focusing the phylogenetic analysis on the 3 lineages we identified helps to reveal a historic pattern of population divergence and subsequent secondary contact within the WC that further reinforces this paradigm.

Within a much broader geographic and evolutionary perspective, our results strongly support a monophyletic origin for the WC hawksbill mtDNA lineage and indicates a degree of differentiation and time frame for its split from eastern Atlantic and Indo-Pacific lineages similar to that reported between the two species in the *Lepidochelys* genus (4.75 mya; 95% HPD: 3.9–5.7 mya). This finding is consistent with the formation of the Panama Isthmus that shaped the deepest phylogenetic divisions in all the extant chelonid species (Bowen et al. 1993; Dutton et al. 1996; Naro-Maciel et al. 2008). Subsequent subdivision within the WC appears to have occurred around 2 mya (95% HPD: 0.5–4.7 mya), and most recently approx 1 mya (95% HPD: 0.3–2.3 mya). This latter timeframe coincides with the mid-Pleistocene glacial cycles, which were characterized by increased climactic fluctuations that would have driven contractions and expansions of nesting habitat in the WC (Diekmann and Kuhn 2002; Reece et al. 2005). These deeper splits in the hawksbill gene tree and evidence for historic population subdivision are similar to those observed for green turtles and hawksbills in the Pacific (Dethmers et al. 2006; Nishizawa et al. 2010), and contrast with the results of a more limited data set for Atlantic hawksbills by Reece et al. (2005) who did not find evidence for population subdivisions during the early Pleistocene. Our results also indicate that there was a general population expansion following a historic bottleneck as did Reece et al. (2005), however, we detected a more recent bottleneck (100 000–300 000 vs. ca. 900 000 years ago in Reece et al. 2005) and this may be due to using a clade by clade analysis instead of pooling data for the entire rookery set. Furthermore, we found that for clade 3, there was both a spatial and demographic expansion. As the Clade 3 haplotypes are primarily confined to the Gulf of Mexico, it is likely that this bottleneck was the result of founders colonizing rookeries and expanding their nesting range in the region. The absence of haplotypes belonging to older gene trees from the other clades may indicate that rookeries were extirpated during one of the glacial cycles of temperature and sea-level fluctuation and the secondary contact evident in other areas of the WC has not occurred in the Gulf of Mexico. This warrants further investigation including larger rookery and haplotype sampling.

Our N_{ef} estimates by clade are much lower than from pooled data per Reece et al. (2005), because our method avoids the artificial inflation of estimates promoted by including divergent lineages. Furthermore, our new current N_{ef} values (6000–9000) is closer to census sizes for the WC hawksbill. Mortimer and Donnelly (2008) compiled a comprehensive survey of available data, which yielded an average of 5248

nesting females per year in the entire Caribbean. Taking into consideration that hawksbill females breed on average every 2.5 years (Beggs et al. 2007) and that the abundances in the Caribbean region suffered a decline of about 77% since the 1900s (Mortimer and Donnelly 2008), historical abundances of breeding Caribbean females 3 generations ago could be estimated at about 57 500 animals which is roughly ten times our N_{ef} values.

Conservation and Management Implications

Additional variation from longer sequences allows greater resolution of nesting stock boundaries. In our case, additional management units (MUs; Moritz 1994) were detected to enhance scientific foundations of management policy, and their inclusion in the baseline for potential WC hawksbill origins will improve the accuracy of stock assignment studies using mixed stock analysis (MSA) to identify the origins of feeding aggregates (Bolker et al. 2003).

First, the nesting populations in Guadeloupe and Nicaragua can be considered as distinct MUs based on the 740bp sequence data (Table 4). These sizeable rookeries (Lagueux et al. 2003; Kamel and Delcroix 2009; Lagueux and Campbell, unpublished data) have not been included as potential source stocks in previous MSA, and this has likely resulted in overestimates of the contributions of the stocks that shared haplotypes with Guadeloupe or Nicaragua (Bowen et al. 2007b; Vélez-Zuazo et al. 2008; Blumenthal et al. 2009; Browne et al. 2010).

Second, splitting the previously identified haplotypes common to several populations reduces the extent of shared haplotypes among potential source stocks for MSA. This should result in tighter confidence limits around the estimated stock contributions (Bolker et al. 2003). For example, the unexpected split between the two proximal Guadeloupe rookeries could be an indication of a local and recent founder event or genetic drift (Table 1), similar to the results found by Browne et al. (2010). Our work detailed here represents a significant update to the baseline data and should be used for future broad-scale analyses.

There remains a need for more extensive sampling of the greater Caribbean's numerous nesting sites to enable a full understanding of stock boundaries within the region. In addition, there are other research priorities that should be considered. For example, work in Puerto Rico has revealed temporal variation in haplotype frequencies among foraging aggregations, which underscores the importance of comprehensive sampling (Vélez-Zuazo et al. 2008); this aspect should be incorporated in future work. In addition, a recent study in Barbados that included more comprehensive spatial sampling of nesting sites revealed significant differences between the populations nesting on beaches on the windward and leeward side of the island (Browne et al. 2010). Unfortunately, sample size limitations prevented us from testing for temporal variability in haplotype frequencies, but this should be a priority for future work in this area.

Further work will also be needed to resolve cases of apparent homogeneity between some rookeries, such as Cuba and

Barbados, since the Cuban rookery has yet to be assessed with the new primers. The additional resolution provided by the longer sequences should be a useful tool in addressing hawksbill stock boundary questions. We recommend the use of the 740bp fragment described here as a minimum standard for future hawksbill mtDNA surveys to improve our understanding of population structure and management needs in the region.

Finally, our results all taken together indicate that hawksbill rookeries should be treated as distinct MUs for conservation purposes, and that loss of one regional rookery in the Gulf of Mexico would result in a notable reduction in genetic diversity for the species. Furthermore, the lack of connectivity between the Holbox, Mexico rookery and the remaining WC indicates that the Gulf of Mexico might not be readily recolonized from eastern Caribbean rookeries over evolutionary timescales.

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Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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