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as revealed by mtDNA

Research Article

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ABSTRACT. Marine migratory species encounter a range of threats as they move through coastal and oceanic zones. Understanding the connectivity and dispersal patterns of such species is critical to their effective conservation. Here we analyzed the temporal genetic composition and the most likely origin of juvenile green turtles foraging at Puerto Manglar and Tortuga Bay, Culebra, Puerto Rico, using mitochondrial DNA control region sequences. We identified 17 haplotypes, of which CM-A3 (51.5%), CM-A5 (19.4%) and CM-A1 (13.6%) were the most common. Haplotype (*h*) and nucleotide (*π*) diversities were 0.680 and 0.008, respectively. There was no evidence of significant variation in the genetic composition of these aggregations throughout seven years (2000-2006), suggesting that relative contributions from source populations did not significantly change during this period. Mixed Stock Analysis (MSA), incorporating 14 Atlantic nesting populations as possible sources, indicated four main contributing stocks to the Culebra foraging grounds: Costa Rica (34.9%), Mexico (29.2%), East Central Florida (13.2%), and Suriname (12.0%). The regional pattern of connectivity among Wider Caribbean rookeries and Culebra was further evidenced by a second MSA using Atlantic Regional Management Units (RMUs) as sources, with 94.1% of the mixed stock attributed to this area. This study addresses the information gap on the connectivity of the green turtle in the North Atlantic, and establishes an important baseline that can be used to determine future changes in stock composition.

Keywords: *Chelonia mydas*, connectivity, mixed stock analysis, mtDNA, foraging ground.

INTRODUCTION

Anthropogenic activities in the world's oceans are leading to a rapid decline of species and marine ecosystems health (Halpern *et al.*, 2008). Marine migratory animals, such as whales (Rasmussen *et al.,* 2007), sharks (Bonfil *et al.*, 2005), seabirds (Catry *et al.*, 2011), and sea turtles (Hays & Scott, 2013), are among the most vulnerable due to the range of threats they encounter during their extensive movements (Lascelles *et al.*, 2014). Understanding the temporal and spatial distribution of these species and the connectivity between geographic areas is therefore essential for an integrated management and the conservation of marine ecosystems.

Sea turtles carry out some of the greatest migrations across ocean basins (Hays & Scott, 2013), going through habitat changes during their lifecycle (Heppell *et al.*, 2002; Bowen & Karl, 2007). The green turtle *Chelonia mydas* immediately after hatching at the beach, reaches the ocean and begins an oceanic period coupled with pelagic habitat and epipelagic feeding (Heppel *et al.,* 2002), which may last 3-5 years in the Greater Caribbean (Reich *et al.*, 2007). During this phase, known as the 'lost years', the distribution and movements of the turtles are poorly known, but they seem to be shaped by a balance between association with oceanic currents (Lahanas *et al.*, 1998; Putman & Naro-Maciel *et al.*, 2013) and directed swimming (Putman & Mansfield, 2015). At 25-35 cm straight-carapace-length (SCL), juveniles recruit to shallow neritic areas and shift to benthic feeding (Heppell *et al.*, 2002; Bolten, 2003). Neritic zones are used as developmental habitats, where turtles spend several

years foraging until reaching a size or maturity stage that triggers them to migrate (Bjorndal *et al.*, 2005a). Sexually mature individuals move periodically from foraging grounds to nesting beaches and mating areas, often separated by hundreds to thousands of kilometres (Bowen *et al.*, 1992; Bowen & Karl, 2007).

The composition of sea turtles at both the nesting beaches and foraging grounds has been assessed with genetic markers. The maternally inherited mitochondrial DNA (mtDNA) has been most widely used (Bowen & Karl, 2007; Lee, 2008; Jensen *et al.*, 2013), revealing that near-shore aggregations of immature green turtles are mixed stocks composed by individuals from multiple nesting colonies, whereas nesting beaches form largely isolated populations (Bowen & Karl, 2007). This structure among rookeries results from the natal philopatry exhibited by marine turtles, in which the reproductive females return to the beaches where they hatched to nest (Meylan *et al.*, 1990), and it enables estimating the sources of turtles sampled at foraging grounds, through the use of Bayesian mixed stock analysis (MSA; Pella & Masuda, 2001). MSA iteratively compares the distribution of haplotype frequencies between a foraging ground and each putative rookery of origin, and may incorporate ecological information such as rookery size, improving model estimates.

In the Greater Caribbean region, unsustainable harvesting of marine turtles during and prior to the 20th century led to the decline of several rookeries. Some of these nesting populations have been recovering over the past decades, following protection from human hazards (*e.g.*, Tortuguero in Costa Rica, Archie Carr Refuge in Florida, Aves Island in Venezuela, Chaloupka *et al.,* 2008, García-Cruz *et al.*, 2015), which consequently should be reflected in the recruitment to juvenile aggregations. MSAs have looked into the origin of foraging grounds in Florida (East Central Florida, Hutchinson Island, St. Joseph Bay and Dry Tortugas and Everglade), Texas, the Bahamas, Barbados, and Nicaragua (Bass & Witzell, 2000; Foley *et al.*, 2007; Naro-Maciel *et al.*, 2012; Proietti *et al.*, 2012; Prosdocimi *et al.*, 2012; Anderson *et al.*, 2013; Naro-Maciel *et al.*, 2016). Developmental foraging habitats are further known from several other areas (*e.g.*, Belize, Bonaire, British and American Virgin Islands, Puerto Rico, St Kitts and Nevis), but they remain genetically uncharacterized.

Of additional importance is the understanding of the temporal variation on genetic composition of mixed stocks. In the Bahamas, variability in the frequency of mtDNA haplotypes of a green turtle juvenile aggregation was detected over a 12-year period and attributed to increased recruitment (Bjorndal & Bolten,

2008). Temporal variability in source contributions has been attributed to very low hatching success at a major source elsewhere (Jensen *et al.*, 2016). Other studies with green turtles in Brazil (Naro-Maciel *et al.*, 2007) and Florida (Naro-Maciel *et al.*, 2016), and with hawksbill turtles in Puerto Rico (Velez-Zuazo *et al.*, 2008), however, found no temporal variation on the genetic composition of juvenile aggregations.

In Puerto Rico, Puerto Manglar and Tortuga Bay at Culebra, are recognized as important developmental habitats for juvenile green turtles (Diez *et al.*, 2010; Patrício *et al.*, 2011, 2014). Turtles as small as 23 cm SCL are known to recruit into these coastal bays, where they spend over a decade, departing before the onset of sexual maturity (Patrício *et al.*, 2011, 2014). Here we investigate the genetic composition of these foraging aggregations during a period of seven years and estimate the most likely origins of these stocks using a MSA, including 14 Atlantic nesting populations as potential sources. This study addresses the information gap on juvenile foraging ground composition in the Caribbean and sets a baseline for the Puerto Rico aggregations, allowing comparisons with future monitoring.

MATERIALS AND METHODS

Study site and sampling

Puerto Manglar (18.30°N, 65.25°W) and Tortuga Bay (18.32°N, 65.23°W) are two foraging grounds for immature green turtles, located at Culebra and Culebrita Islands, respectively, within the boundaries of a critical habitat for the green turtle, designated by the Endangered Species Act (NMFS-NOAA, 1998) in Puerto Rico (see Fig. 1 in Patrício *et al.*, 2011). The Department of Natural and Environmental Resources of Puerto Rico (DNER-PR) has conducted a capture-mark-recapture program at these sites, since 1997. From 2000 to 2006 we collected samples from 103 green turtles foraging in these bays [2000 (18), 2001 (16), 2002 (2), 2003 (17), 2004 (13), 2005 (25), 2006 (12)]. Turtles were captured with an entanglement net (200 m long, 5 m deep, nylon twine, 25 cm stretch mesh size) deployed for ~1 h sets at <5 m depth, with the help of a motor boat. Swimmers snorkelled continually along the net to locate and disentangle trapped turtles. Turtles were kept in the shade and covered with wet towels while captive and until processing. Handling time averaged 15 min per individual, after which turtles were released close to their capture location. Tissue samples were collected from the shoulder area using a disposable biopsy punch (4-6 mm diameter, Acuderm®). Samples were preserved in 95% ethanol or salt-saturated 20% DMSO-20% EDTA and stored at room temperature. SCL of sampled individuals was measured

with Haglof tree calipers to the nearest 0.1 cm. All turtles were applied a unique ID tag in both front flippers to avoid misidentification and sample duplication.

Sequencing and haplotype assignment

DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen) following manufacturer's instructions, and eluted in a final volume of 50 µL per sample. DNA concentrations were quantified with a spectropho-tometer (NanoDrop® ND-3300) and a 735 bp fragment of the mtDNA control region was amplified by Polymerase Chain Reaction (PCR) with primers LTEi9 and H950 (Abreu-Grobois *et al.*, 2006). Amplifications were performed in a total volume of 10 µL, with 1 µL genomic DNA at a concentration of ~10 ng µL^{-1}, 4.0 µL of Qiagen Taq Master Mix, 0.5 µM of each primer at 10 µM and 2.0 µL MilliQ water. PCR started with an initial denaturing step of 5 min at 94ºC, followed by 30 cycles of 30 s at 94ºC, 30 s at 52ºC, and 1 min at 72ºC, with a final hold at 72ºC for 5 min. All PCR reactions included positive and negative controls. PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced in both forward and reverse directions using a BigDye Terminator v.3.1 (Bioanalytical Instruments) and the automated sequencer station ABI 3130xl (Applied Biosystems) at the Sequencing and Genotyping Facility of the University of Puerto Rico, Río Piedras. Sequences were assembled and aligned by eye using Sequencher 4.5 (Gene Codes). To identify unique haplotypes and estimate absolute haplotype frequencies we used DNAspv4.10 (Rozas *et al.*, 2003). Haplotypes were identified using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and named following the standardized nomenclature of the Archie Carr Center for Sea Turtle Research.

Diversity estimates

Haplotype (*h*) and nucleotide diversities (*π*), pairwise genetic distances among groups (F_{ST}), and exact tests of differentiations (Raymond & Rousset, 1995) were estimated using Arlequinv 3.1 (Excoffier & Lischer, 2010) for two sets of groups: 1) sample years at our study sites (n = 6), and 2) Atlantic green turtle foraging grounds (n = 18, Fig. 1). A false discovery rate (FDR) correction, following Narum (2006), was applied to calculate the most fitting threshold for the *P*-value significance, considering the number of comparisons involved in the analysis, under an expected original threshold of $P < 0.05$. The sample size for 2002 was too small (*i.e.*, n = 2) for robust statistic comparisons among years, so it was excluded from the temporal

analysis. We truncated the DNA fragments to 491 bp length, the fragment historically explored and for which most genetic information is currently available, to compare diversity estimates with other foraging aggregations.

Geographic variability and genetic diversity

To investigate how mithocondrial control region diversity is partitioned among foraging aggregations, we conducted a spatial analysis of molecular variance (SAMOVA, Dupanloup *et al.*, 2002), incorporating geographic positions obtained through Google Earth, and using 100 simulated annealing processes. This analysis defines geographic groups that are maximally differentiated (rather than defining *a priori* groupings). The F_{CT} statistic from AMOVA (calculated *a posteriori*) was then compared among different values of groups (K), ranging from 2 to 18 foraging grounds, to assess the most likely number of K, corresponding to the highest F_{CT} (Dupanloup *et al.*, 2002). Additionally, genetic distances between foraging sites were included in a principal coordinate analysis (PCoA) using the package Genalex 6.5.0.1 (Peakall & Smouse, 2012), to plot variability in a two-dimensional space.

Mixed stock analysis (MSA)

The most likely origin of the studied aggregations was estimated through a "one-to-many" MSA using BAYES (Pella & Masuda, 2001). We compiled the available genetic information from green turtle Atlantic nesting populations and used it as baseline information for the MSA (See Fig. 1 for sites included in this study, site abbreviations, and literature sources, and Table 1 for genetic composition). Rookery size, defined as the number of nesting females per rookery (Seminoff *et al.*, 2015), was used to establish weighted priors. Previous studies have shown that there is significant structure among most of the genetically characterized Atlantic green turtle rookeries (Bolker *et al.*, 2007, Shamblin *et al.*, 2012, 2014), supporting the applicability of a MSA. There is however a lack of genetic differentiation at the mtDNA control region between some individual rookeries (*e.g.*, Suriname and Aves Island, Naro-Maciel *et al.*, 2016), so we also ran a MSA pooling the individual rookeries into Regional Management Units (RMUs, Wallace *et al.*, 2010), which group multiple nesting populations based on their genetic similarities, for conservation management. Following Naro-Maciel *et al.* (2016), the RMUs were defined as: 1) Northwest Atlantic - EcFL, SFL, MEX, CUB, CR; 2) Central Atlantic - BUC, AV, SUR; and 3) South and East Atlantic - RC/FN, ASC, TRI, GB, BIO, STP. Four independent chains with different starting points were run for 30,000 iterations, with a burn-in of 15,000 steps. We used the Gelman-Rubin diagnostic to assess conver-
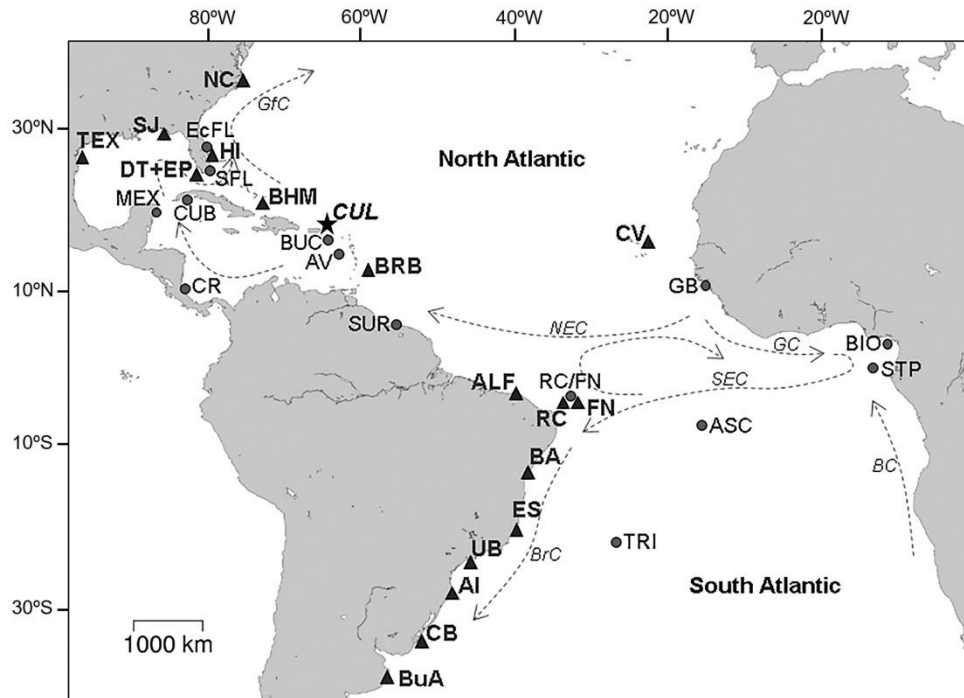
Figure 1. Atlantic green turtle (*Chelonia mydas*) foraging grounds (n = 18, dark triangles and black star for study site) and nesting populations (n =14, gray circles) included in this study, with respect to major ocean currents: GfC: Gulf Current, NEC: North Equatorial Current, SEC: South Equatorial Current, BrC: Brazil Current, GC: Guinea Current, BgC: Benguela Current. Nesting populations: EcFL and SFL: Florida, USA (Shamblin *et al*., 2014); CUB: southwest Cuba (Ruiz-Urquiola *et al*., 2010); MEX: Quintana Roo, Mexico (Encalada *et al*., 1996); CR: Tortuguero, Costa Rica (Bjorndal *et al*., 2005b; Encalada *et al*., 1996); SUR: Matapica and Galibi, Suriname (Encalada *et al*., 1996; Shamblin *et al*., 2012); AV: Aves Island (Lahanas *et al*., 1998, 1994; Shamblin *et al*., 2012), Venezuela; BUC: Buck Island (Shamblin *et al*., 2012); RC/FN: Rocas Atoll and Fernando Noronha (Bjorndal *et al*., 2006; Encalada *et al*., 1996), Brazil; ASC: Ascension Island (Encalada *et al*., 1996; Formia *et al*., 2007); TRI: Trindade Island, Brazil (Bjorndal *et al*., 2006); GB: Poilão, Guinea-Bissau (Patrício *et al*., 2017); BIO: Bioko Island, Equatorial Guinea (Formia *et al*., 2006); STP: Sao Tome and Principe (Formia *et al.*, 2006). Foraging grounds: NC: North Carolina (Bass *et al*., 2006), HI: Hutchinson Island, Florida (Bass & Witzell, 2000), DT+EP: Dry Tortugas + Everglades Park, Florida (Naro-Maciel *et al*., 2016), SJ: St. Joseph Bay, Florida (Foley *et al*., 2007), TEX: Texas (Anderson *et al*., 2013), USA; BHM: Bahamas (Lahanas *et al*., 1998), CUL: Culebra, Puerto Rico (this study), BRB: Barbados (Luke *et al*., 2004), ALF: Almofala, Brazil (Naro-Maciel *et al*., 2007), RC: Rocas Atoll, Brazil (Naro-Maciel *et al*., 2012), FN: Fernando Noronha, Brazil (Naro-Maciel *et al*., 2012), BA: Bahia, Brazil (Naro-Maciel *et al*., 2012), ES: Espirito Santo, Brazil (Naro-Maciel *et al*., 2012), UB: Ubatuba, Brazil (Naro-Maciel *et al*., 2007), AI: Arvoredo Island, Brazil (Proietti *et al*., 2012), CB: Cassino Beach, Brazil (Proietti *et al*., 2012), BuA, Buenos Aires, Argentina (Prosdocimi *et al*., 2012), CV: Cape Verde (Monzón-Argüello *et al*., 2010).

gence of the chains to the posterior distribution, assuming that there was no evidence of non-convergence at values <1.2 (Pella & Masuda, 2001).

RESULTS

At Puerto Manglar (n = 60) mean SCL was 47.4 ± 8.8 cm (mean ± SD, range: 32-70.9 cm, Fig. 2), and at Tortuga Bay (n = 43) it was 44.7 ± 11.0 cm (mean ± SD, range: 28.4-69.8 cm, Fig. 2). There was no significant difference in SCL distribution between the two groups (t_{101}= 1.3832, P = 0.1696).

We detected 17 polymorphic sites at the 735 bp mtDNA fragment, one transversion, 16 transitions and one insertion (position 617), defining 17 haplotypes, 13 of them previously described (Supplemental Table 1). After truncating the sequences the total number of haplotypes dropped to 10 (Table 1). In both aggregations the haplotype CM-A3 was dominant (PM: 43%; TB: 63%), followed by haplotypes CM-A5 (PM: 22%; TB: 16%), CM-A1 (PM: 15%; TB: 12%), and CM-A8 (PM: 7%; TB: 5%). We also identified rare haplotypes with frequencies of 1-3%: CM-A2, CM-A16, CM-A17, CM-A18, CM-A27 and an orphan haplotype, CM-A26,
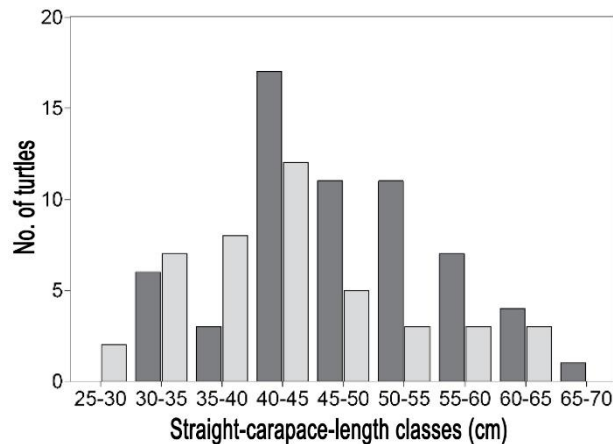
Figure 2. Straight-carapace-length (SCL, cm) distributions for immature green turtles (*Chelonia mydas*) captured between 2000 and 2006 at Puerto Manglar (n = 60, dark gray) and Tortuga Bay (n = 43, light gray) foraging grounds, Puerto Rico.

not yet reported in a nesting population, emphasizing that some stocks still lack genetic studies or have not yet been adequately sampled. A randomized Chi-square ($\chi^2 = 6.05$, $P = 0.89$) and an exact test of differentiation ($P = 0.88$) indicated no significant genetic structure between the two aggregations, so these were pooled for further analyses, referred to henceforth as the Culebra foraging ground. We found no significant temporal variation in the haplotype composition of the Culebra foraging ground among sampling years over seven-year period (Table 2). There seems to be an increase in haplotype CM-A5 with time, however (Supplemental Fig. 1).

The haplotype (*h*) and nucleotide (π) diversities at Culebra foraging grounds were comparable to those of Atlantic green turtle aggregations (Table 3). Culebra was significantly different from all other foraging sites except the Bahamas (Table 4).

The SAMOVA suggested that the 18 foraging aggregations were partitioned into two or three main groups, with $F_{CT} = 0.7061$ for K = 2, and $F_{CT} = 0.7074$ for K = 3. The estimates of F_{CT} decreased faster as K increased, after K = 3 (Supplemental Fig. 2). Because the percentage variation between populations within groups increased from 1.5% for K = 3 to 2.5% for K = 2 (Supplemental Fig. 2) by including Barbados with the south Atlantic foraging grounds, we consider that K = 3 is a better grouping. This was consistent with the PCoA. The SAMOVA (K = 3) and the PCoA separated foraging areas geographically, highlighting three groups: 1) all South American foraging grounds and Cape Verde, 2) Northwest Atlantic foraging grounds, and 3) Barbados (Fig. 3). Using this *a priori* grouping

in the AMOVA, a highly significant structure was observed among the groups ($F_{ST} = 0.7289$, $P < 0.001$).

The MSA using RMUs as potential sources estimated that 77.9% of the green turtles foraging at Culebra recruit from the Northwest Atlantic RMU (95% CI: 68.4-86.6%), 16.2% from the Central Atlantic RMU (95% CI: 8.4-25.2%) and 5.9% from the South and East Atlantic RMU (95% CI: 2.1-11.2%) (Fig. 4a). The MSA using individual nesting populations estimated that 34.9% of the Culebra turtles originated from Tortuguero, Costa Rica (95% CI: 1.4-58.3%); 29.2% from Mexico (95% CI: 5.8-61.8%); 13% from East Central Florida (95% CI: 0-60.7%); 12% from Suriname (95% CI: 0-24.2%), 3% from South Florida (95% CI: 0-20.1%), 3% from Cuba (95% CI: 0-21.8%) and 3.5 % from Guinea-Bissau (95% CI: 0-9.9%) (Fig. 4b, and Supplemental Table 2).

DISCUSSION

Understanding the links between developmental habitats and the source populations of migratory species is critical to assess threats at their different life stages, and develop effective conservation policies. Here we analyzed the genetic composition of two important developmental aggregations for green turtles in the Caribbean (Culebra, Puerto Rico), over a period of seven years, and predicted the most likely connectivity of these aggregations to Atlantic nesting populations, using mtDNA control region sequences and a MSA, improving our understanding on the movements of green turtles in the North Atlantic.

Genetic structure among foraging aggregations
The similarity in the genetic composition of Tortuga Bay and Puerto Manglar suggests that there is no differential recruitment between the two foraging grounds, which was expected given that these are only 2 km apart. There was also no significant genetic differentiation between Culebra and the Bahamas. This foraging ground also has major contributions from Northwest Atlantic rookeries, but not from Central Atlantic rookeries (Putman & Naro-Maciel, 2013), contrary to what we estimated for Culebra. At greater distances however, there is structure among foraging grounds, and we found two major groups, represented by the northwest Atlantic and the south and east Atlantic. The Barbados mixed stock was distinct from both groups, as it receives equal contributions from both north and south Atlantic nesting populations (Luke *et al.*, 2004), potentially due to its position relative to the coalescence of the North Equatorial and South Equatorial currents (Luke *et al.*, 2004).

Table 1. mtDNA haplotype frequencies at the study site and at 14 Atlantic green turtle nesting populations, with total number of samples and haplotypes per area, and total number of nesting females at rookeries. See Fig. 1 for site abbreviations.

Haplotypes	CUL[a]	MEX[b]	EcFL[b,c]	SFL[c]	CR[d,e]	AV[e,f,g]	CUB[h]	SUR[b,g]	BUC[g]	TRI[i]	RC/N[b,i]	ASC[b,j,k]	GB[l]	BIO[j]	STP[j]
						Nesting populations									
CM1	14	7	197	27			3								
CM2	1		7	4											
CM3	53	5	92	127	395	5	16	1							
CM4					1										
CM5	20	1	2	4	32	62		68	45						1
CM6								3				11		5	1
CM7								1							
CM8	6		1							67	50	204	170	45	17
CM9										19	7	9			
CM10											2	5			
CM11										1	1				
CM12												5			
CM13			7	2											
CM15		1													
CM16	2	1	2	1					4						
CM17	1	2		2											
CM18	3	3	1	1											
CM20					2										
CM21					3										
CM23										6		1			
CM24										1		7			
CM25											3	1			
CM26	1														
CM27	2						1								
CM28			2	3			1								
CM32										4	1	1			
CM33										1					
CM35															1
CM36															3
CM37															1
CM38															2
CM39												1			
CM42													1		
CM44												1			
CM45												1			
CM46												2			
CM48							5								
CM50												1			
CM53				3											
CM56							1								
CM57							1								
Sample size	103	20	311	174	433	67	28	73	49	99	69	245	171	50	26
Haplotype no.	10	7	9	10	5	2	7	4	2	7	7	13	2	2	7
Rookery size[n]		18,257	4,990	3,302	131,751	2,833	2,226	13,417	63	2,016	345	13,417	29,016	850	376

[a]This study, [b]Encalada *et al.* (1996), [c]Shamblin *et al.* (2014), [d]Bjorndal *et al.* (2005), [e]Lahanas *et al.* (1998), [f]Lahanas *et al.* (1994), [g]Shamblin *et al.* (2012), [h]Ruiz-Urquiola *et al.* (2010), [i]Bjorndal *et al.* (2006), [j]Formia *et al.* (2006), [k]Formia *et al.* (2007), [l]Patrício *et al.* (2017), [n]Seminoff *et al.* (2015).

Table 2. Sample size (n), total number of haplotypes (hap), and haplotype (*h*) and nucleotide diversities (π) per year, at Culebra foraging ground (Puerto Rico), for immature green turtles, throughout a seven year period, and pairwise comparisons among sampling years: exact test *P*-values ($P > 0.05$) in the above diagonal and F_{ST} values in the below diagonal.

Year	n	Hap	*h*	π	Year					
					2000	2001	2003	2004	2005	2006
2000	18	5	0.743 ± 0.089	0.007 ± 0.004		0.67	0.77	0.55	0.72	0.700
2001	16	5	0.608 ± 0.130	0.005 ± 0.003	-0.04		0.51	0.16	0.46	0.278
2003	17	5	0.684 ± 0.099	0.009 ± 0.005	-0.03	0.01		0.74	0.97	0.898
2004	13	7	0.846 ± 0.076	0.011 ± 0.007	0.03	0.11	-0.03		0.23	1.000
2005	25	5	0.607 ± 0.093	0.008 ± 0.005	-0.04	-0.02	-0.04	0.01		0.658
2006	12	5	0.758 ± 0.093	0.012 ± 0.007	0.04	0.12	-0.04	-0.08	0.01	

Table 3. Sample size (n), haplotype number (hap) and haplotype (*h*) and nucleotide (*π*) diversity estimates ± SD of Atlantic green turtle (*Chelonia mydas*) foraging grounds (n = 18), using a fragment of 491 bp of the control region of the mitochondrial DNA as a marker. The study population is represented in bold.

Juvenile foraging grounds	n	hap	*h*	(*π*)
Culebra, Puerto Rico[a]	**103**	**10**	**0.680 ± 0.040**	**0.008 ± 0.005**
North Carolina, USA[b]	106	12	0.729 ± 0.030	0.005 ± 0.003
Hutchinson island, FL, USA[c]	62	6	0.486 ± 0.067	0.003 ± 0.002
St. Joseph, FL, USA[d]	255	13	0.711± 0.022	0.004 ± 0.003
Dry Tortugas and Everglades, FL, USA[e]	138	15	0.715 ± 0.0301	0.005 ± 0.003
Texas, USA[f]	282	15	0.606 ± 0.019	0.002 ± 0.002
Bahamas[g]	79	6	0.370 ± 0.065	0.006 ± 0.004
Barbados[h]	60	8	0.773 ± 0.028	0.010 ± 0.006
Ubatuba, Brazil[i]	113	10	0.446 ± 0.056	0.002 ± 0.002
Almofala, Brazil[i]	117	13	0.717 ± 0.031	0.007 ± 0.004
Rocas, Brazil[j]	101	8	0.688 ± 0.036	0.005 ± 0.003
Fernando Noronha, Brazil[j]	117	12	0.650 ± 0.028	0.004 ± 0.003
Bahia, Brazil[j]	45	6	0.648 ± 0.053	0.002 ± 0.002
Espirito Santo, Brazil[j]	157	9	0.595 ± 0.031	0.003 ± 0.002
Arvoredo Island, Brazil[k]	115	12	0.583 ± 0.045	0.002 ± 0.002
Cassino Beach, Brazil[k]	101	12	0.586 ± 0.050	0.003 ± 0.002
Buenos Aires, Argentina[l]	93	9	0.553 ± 0.051	0.002 ± 0.002
Cape Verde[m]	44	5	0.588 ± 0.045	0.004 ± 0.003

[a]This study, [b]Bass *et al.* (2006), [c]Bass & Witzell (2000), [d]Foley *et al.* (2007), [e]Naro-Maciel *et al.* (2016), [f]Anderson *et al.* (2013), [g]Lahanas *et al.* (1998), [h]Luke *et al.* (2004), [i]Naro-Maciel *et al.* (2007), [j]Naro-Maciel *et al.* (2012), [k]Proietti *et al.* (2012), [l]Prosdocimi *et al.* (2012), [m]Monzón-Argüello *et al.* (2010).

Regional connectivity among Culebra and Wider Caribbean populations

The MSAs indicated that the Culebra aggregations originate from multiple rookeries within the Wider Caribbean region. This strong regional connectivity agrees with the 'closest to home' hypothesis, where immature turtles tend to move to and settle in foraging grounds closest to their natal beach after recruiting to neritic habitats (Bowen *et al.*, 2004; Bolker *et al.*, 2007). Similar patterns of regionalized recruitment have already been observed in Atlantic green turtles (Bass *et al.*, 2006; Bolker *et al.*, 2007; Naro-Maciel *et al.*, 2012) and in other marine turtle species (Bowen & Karl, 2007). However, this pattern may be influenced by the geographic position of foraging areas and nesting beaches relative to major oceanic currents (Luke *et al.*, 2004). The connectivity within the Wider Caribbean estimated in the MSA is supported by several tag returns from foraging and nesting adult turtles (Fig. 5). Most of these tags were recovered at Nicaragua (n = 8), at foraging grounds long known to be used by the nesting population of Tortuguero (*i.e.*, Miskito Cays, Carr & Ogren, 1960; Bjorndal, 1980), but also at Venezuela (n = 1), Colombia (n = 1), and Florida (n = 1). In the latter, a turtle first tagged as a juvenile at Tortuga Bay in 1997, was found nesting in

2014 (Bagley, *pers. comm.*), further confirming this connectivity. Interestingly, there was also a tag return in 2006 from the north of Brazil (State of Ceará, >3500 km, Lima *et al.*, 2008), so more distant links can exist.

Temporal variability

Throughout the seven years of this study we could not detect a significant variation on the frequency of the mtDNA haplotypes at the Culebra aggregation, which could suggest that there were no changes in the overall contributions from the major source populations (*i.e.*, Costa Rica, Mexico, East Central Florida and Suriname). These results are not conclusive however, because our annual sample size may have been too small to detect significant change. We did observed a slight increase in the frequency of haplotype CM-A5, which could potentially be associated with the positive trend in population growth at rookeries where this is the dominant haplotype, *i.e.,* Suriname and Aves Island (García-Cruz *et al.*, 2015; Turny, *pers. comm.*). At Puerto Manglar, a positive trend on abundance with a mean annual increase of 10.9% was observed over the course of 15 years (1998-2012, Patrício *et al.*, 2014), more accentuated from 2006, owing to increased recruitment. This reflects the positive trend in the source populations (Chaloupka *et al.*, 2008), which may

Table 4. Pairwise exact test P-values (above diagonal) and pairwise F_{ST} values (below diagonal) among 18 Atlantic green turtle foraging aggregations, based on ~490 bp sequences of the control region of the mtDNA. The study site is in bold, and abbreviations follow those in Figure 1. Asterisks indicate statistically significant comparisons (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). i) prior to corrections, in the low diagonal, ii) after FDR correction, in the above diagonal. Non-significant values, after FDR correction, are marked in bold (for a $P < 0.05$, FDR = 0.009; $P < 0.01$, FDR = 0.002, for a $P < 0.015$, FDR = 0.000; Narum, 2006).

|	NC	HI	DT+EP	SJ	TEX	BHM	CUL	BRB	ALF	RC	FN	BA	ES	UB	AI	CB	BuA	CV
NC		**0.036**	**0.315**	**0.018**	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
HI	**0.035***		0.009*	0.000***	0.000***	0.009*	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
DT+EP	**0.001**	0.009*		**0.613**	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
SJ	**0.012***	0.028***	**-0.001**		0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
TEX	0.050***	0.082**	0.029***	0.018***		0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
BHM	0.051***	0.050***	0.064***	0.103***	0.206***		**0.063**	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
CUL	0.043***	0.100***	0.073***	0.116***	0.203***	**0.022**		0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
BRB	0.227***	0.324***	0.286***	0.364***	0.488***	0.179***	0.079***		0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
ALF	0.552***	0.632***	0.595***	0.649***	0.733***	0.508***	0.395***	0.165***		**0.198**	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
RC	0.632***	0.731***	0.676***	0.717***	0.799***	0.605***	0.486***	0.258***	**0.005**		0.000***	**0.081**	0.000***	0.000***	0.009*	0.000***	0.000***	0.000***
FN	0.689***	0.768***	0.716***	0.751***	0.823***	0.654***	0.543***	0.333***	0.054***	0.030***		**0.171**	0.000***	0.000***	0.000***	0.000***	0.000***	**0.685**
BA	0.722***	0.833***	0.746***	0.780***	0.863***	0.684***	0.556***	0.346***	0.060***	**0.022**	**0.015**		**0.919**	0.000***	**0.270**	**0.081**	**0.225**	**0.027**
ES	0.754***	0.834***	0.772***	0.79***	0.856***	0.731***	0.623***	0.431***	0.073***	0.026***	0.024***	**-0.011**		0.000***	**0.117**	0.009	**0.126**	0.000***
UB	0.749***	0.846***	0.768***	0.787***	0.860***	0.728***	0.608***	0.413***	0.075***	0.044***	0.119***	0.065***	0.054***		**0.036**	**0.117**	**0.054**	0.000***
AI	0.744***	0.835***	0.764***	0.786***	0.857***	0.720***	0.605***	0.409***	0.069***	0.028***	0.060***	**0.004**	**0.007**	**0.011**		**0.901**	**0.964**	0.000***
CB	0.741***	0.833***	0.762***	0.785***	0.858***	0.716***	0.599***	0.404***	0.075***	0.037***	0.073***	**0.015**	0.017**	**0.007**	**-0.006**		**0.901**	0.000***
BuA	0.744***	0.841***	0.764***	0.787***	0.862***	0.719***	0.599***	0.403***	0.071***	0.031***	0.062***	**0.006**	**0.008**	**0.013**	**-0.008**	**-0.007**		0.000***
CV	0.682***	0.785***	0.712***	0.754***	0.842***	0.633***	0.507***	0.29***	0.060***	0.048*	**-0.009**	0.047*	0.060***	0.200***	0.116***	0.131***	0.120***	
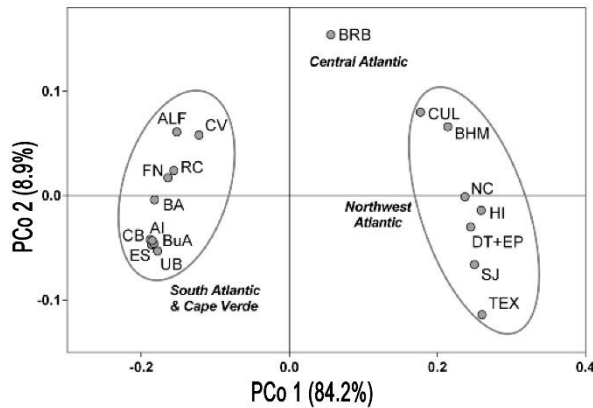
Figure 3. PCoA of 18 Atlantic green turtle (*Chelonia mydas*) foraging grounds using F_{ST} genetic distances inferred from control region mitochondrial DNA haplotypes. The percentage of the variability explained by each coordinate is shown in brackets. Foraging grounds: NC: North Carolina, USA; EcFL: East Central Florida, USA; BHM: Bahamas; CUL: Culebra, Puerto Rico; BRB: Barbados; ALF: Almofala, RC: Rocas Atoll, FN: Fernando Noronha, BA: Bahia, ES: Espirito Santo, UB: Ubatuba, Brazil, AI: Arvoredo Island, and CB: Cassino Beach, Brazil; BuA, Buenos Aires, Argentina; CV: Cape Verde.

lead to changes in the relative contributions from Atlantic rookeries to the Culebra aggregation, particularly if they are not all recover at the same pace.

Impact for nesting and breeding recruitment

Both Tortuga Bay and Puerto Manglar foraging grounds are recruitment sites for post-pelagic individuals, where minimum sizes found are 22.8 and 29.8 cm SCL, respectively (Diez *et al*., 2010). A long-term capture-mark-recapture (CMR) program has revealed that immature turtles remain in these bays for several years (*ca.* 10 to 17 years, Patrício *et al*., 2014), and that larger immature turtles (>65 cm SCL) permanently emigrate, potentially to subadult foraging sites closer to their breeding grounds (Patrício *et al*., 2011). As turtles spend such a long period of their early life at these developmental sites, mortality there can impact the multiple rookeries to which they are linked. Juvenile green turtles at Culebra's aggregations have high survival probability (0.83; CI$_{95\%}$ = 0.79-0.87, Patrício *et al*., 2011), comparable to estimates found for juvenile mixed stocks in areas virtually free of human impacts (Bjorndal *et al*., 2003; Chaloupka & Limpus, 2005). Occasional stranding's of immature green turtles with evidence of boat collisions or of fibropapilloma tumors have occurred; otherwise no direct hazards for green turtles are known at the study sites. Habitat degradation, however, may have a negative impact, as both coastal urban development and recreational boats continue to increase in the area. Fibropapillomatosis (FP) is endemic to Culebra's aggregations and in 2003 disease prevalence reached 75% at the most affected
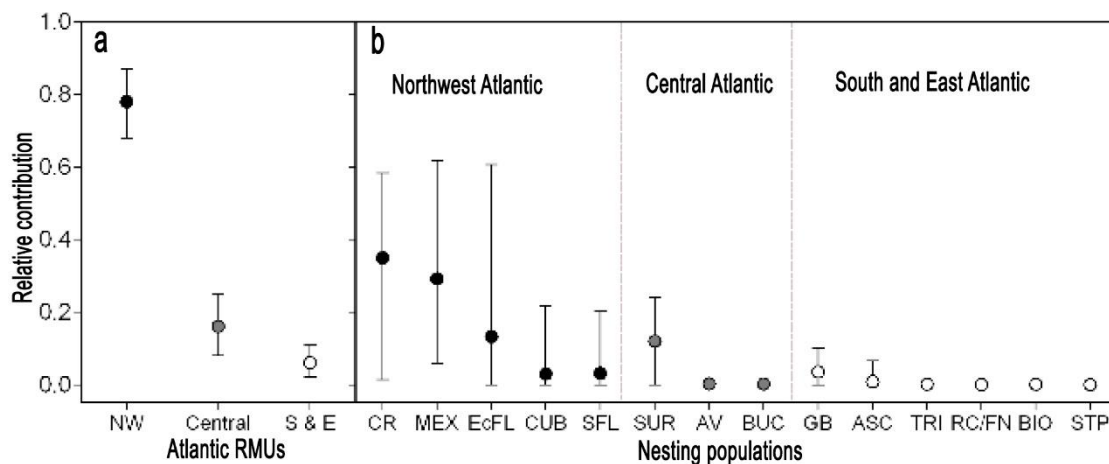

Figure 4. Mean proportion and 95% confidence intervals (error bars) of green turtles (*Chelonia mydas*) foraging at Culebra, Puerto Rico, attributed to a) three Atlantic Regional Management Units (RMUs): Northwest Atlantic (CR, MEX, EcFL, CUB, SFL), Central Atlantic (SUR, AV, BUC) and South and East Atlantic (GB, ASC, TRI, RC/FN, BIO, STP), and b) each of 14 Atlantic nesting populations, estimated by a mixed-stock-analysis. Nesting populations: CR: Tortuguero, Costa Rica; MEX: Quintana Roo, Mexico; EcFL: East Central Florida, USA; SUR: Matapica and Galibi, Suriname; CUB: southwest Cuba; SFL: Florida, USA; GB: Poilão, Guinea-Bissau; ASC: Ascension Island; AV: Aves Island, Venezuela; BUC: Buck Island; TRI: Trindade Island, Brazil; RC/FN: Rocas Atoll and Fernando Noronha, Brazil; BIO: Bioko Island, Equatorial Guinea; STP: Sao Tome and Principe.
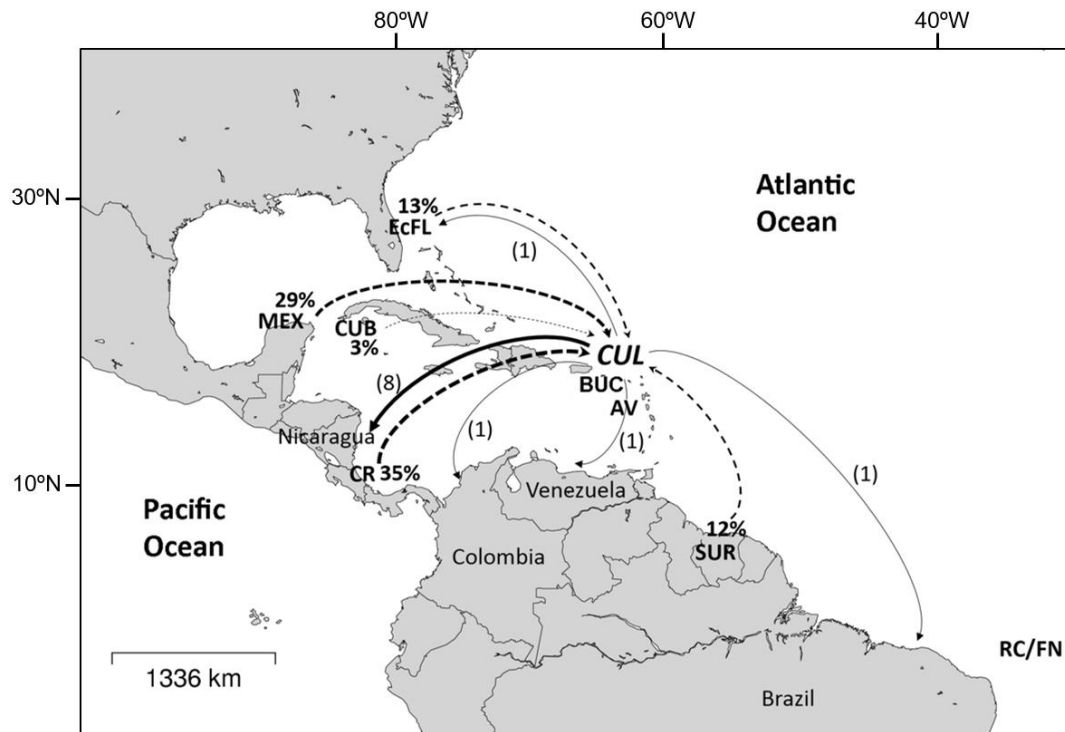
Figure 5. Map showing green turtle (*Chelonia mydas*) rookeries in the wider Caribbean region that contribute to the Culebra (Puerto Rico) foraging aggregations (dashed arrows, contributions ≥3%), and locations of tag returns from turtles resident at Culebra (solid arrows). Mean percentage contributions by the different nesting populations, as estimated through Bayesian mixed-stock-analysis (MSA) are indicated in bold, as well as number of tag returns (in parenthesis). Note: the pathways shown are not indicative of migratory corridors. EcFL: East Central Florida, SFL: South Florida, USA; CUB: southwest Cuba; MEX: Quintana Roo, Mexico; CR: Tortuguero, Costa Rica; AV: Aves Island, Venezuela; SUR: Matapica and Galibi, Suriname; RC/FN: Rocas Atol and Fernando Noronha, Brazil; and CUL: Culebra foraging aggregation (Map created using www.seaturtle.org/maptool).

foraging site (*i.e.*, Puerto Manglar, Diez *et al.*, 2010). It was shown, however, that FP did not affect survival rates (Patrício *et al.*, 2011), and that individual recovery was likely (Patrício *et al.,* 2016).

CONCLUSIONS

Green turtles, once abundant in the Caribbean, faced major population decline of possibly 99%, since the arrival of European (Jackson, 1997). Thanks to conservation efforts of the past decades, major green turtle populations worldwide are now rapidly recovering (Chaloupka *et al.*, 2008). This has been particularly noticeable in the wider Caribbean region, where long-term data allows for robust abundance trend estimates of major populations, *e.g.,* Costa Rica, Florida, and Mexico (Seminoff *et al.*, 2015). A positive abundance trend was also detected at Puerto Manglar, as mentioned earlier (Patrício *et al.,* 2014). Turtles are however still harvested in some regions in the wider

Caribbean (Humber *et al.*, 2014). Most notably at Nicaragua there is a large legal artisanal fishery of green turtles aimed for local consumption (Humber *et al.*, 2014; Lagueux *et al.*, 2014), but additional commercialization of turtle meat continues to occur due to lack of law enforcement, and this fishery was estimated to take *ca.* 8000 turtles per year, and considered to be unsustainable (Lagueux *et al.*, 2014). The majority of tag returns from the Culebra aggregation came from Nicaragua, which poses a conservation paradox if efforts are conducted to protect these juvenile aggregations but unsustainable harvesting at later stages of their life occurs elsewhere. Our study emphasizes, therefore, the widely recognized need for a comprehensive regional conservation strategy (Wallace *et al.*, 2011).

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**Supplemental Table 1.** Genetic composition of the foraging aggregation of green turtles at Culebra, Puerto Rico, based on the long version of mtDNA haplotypes (735 bp). Haplotype names for long fragments are based on nomenclature established and suggested by the Archie Carr Center for Sea Turtle Research (accstr.ufl.edu/ resources/mtdna-sequences/). Only the new haplotypes reported in our study have been designated a sequence number and deposited in Genbank.

Haplotype		Site	
Short fragment	735 bp	CUL[a]	Accession No.
CM-A1	CM-A1.1	7	
	CM-A1.2	3	
	CM-A1.4	4	
CM-A2	CM-A2.1	1	
CM-A3	CM-A3.1	33	
	CM-A3.X	20	MF315093
CM-A5	CM-A5.1	15	
	CM-A5.2	5	
CM-A8	CM-A8.1	4	
	CM-A8.X	2	MF315094
CM-A16	CM-A16.1	1	
	CM-A16.X	1	MF315095
CM-A17	CM-A17.1	1	
CM-A18	CM-A18.2	2	
	CM-A18.X	1	MF315096
CM-A26	CM-A26.1	1	
CM-A27	CM-A27.1	2	
	Sample size	103	
	Haplotype no.	17	

Supplemental Table 2. Relative contributions of 14 green turtle (*Chelonia mydas*) Atlantic rookeries (sources) to a juvenile aggregation at Culebra, Puerto Rico, estimated through a Bayesian mixed stock analysis. Nesting populations by contribution (largest to lowest): CR: Tortuguero, Costa Rica; MEX: Quintana Roo, Mexico; EcFL: East Central Florida, USA; SUR: Matapica and Galibi, Suriname; CUB: southwest Cuba; SFL: Florida, USA; GB: Poilão, Guinea-Bissau; ASC: Ascension Island; AV: Aves Island, Venezuela; BUC: Buck Island; TRI: Trindade Island, Brazil; RC/FN: Rocas Atol and Fernando Noronha, Brazil; BIO: Bioko Island, Equatorial Guinea; STP: Sao Tome and Principe.

Source	Mean	CI: 97.5%	CI: 2.5%	SD	Median
CR	0.349	0.583	0.014	0.155	0.380
MEX	0.292	0.618	0.058	0.144	0.275
EcFL	0.132	0.607	0.000	0.181	0.034
SUR	0.120	0.242	0.000	0.071	0.132
CUB	0.030	0.218	0.000	0.064	0.000
SFL	0.031	0.201	0.000	0.059	0.000
GB	0.035	0.099	0.000	0.030	0.033
ASC	0.008	0.066	0.000	0.018	0.000
AV	0.002	0.012	0.000	0.014	0.000
BUC	0.002	0.000	0.000	0.019	0.000
BIO	0.001	0.001	0.000	0.006	0.000
TRI	0.001	0.003	0.000	0.005	0.000
RCN	0.000	0.000	0.000	0.002	0.000
STP	0.000	0.000	0.000	0.003	0.000
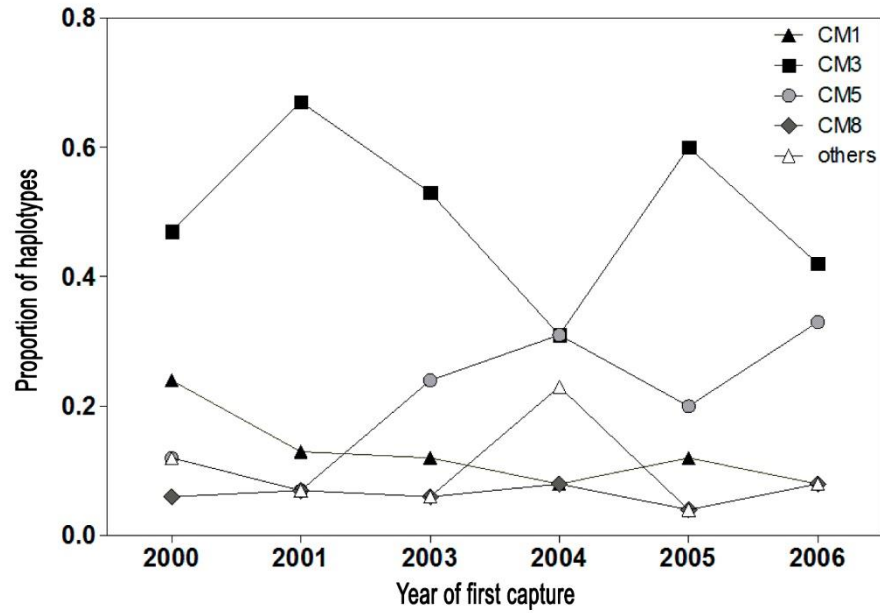
Supplemental Figure 1. Proportion of green turtle (*Chelonia mydas*) control region mitochondrial DNA haplotypes for 6 years at a juvenile foraging aggregation, Culebra, Puerto Rico. Haplotypes that were not present in all of the annual samples were combine in 'others' (n = 5).
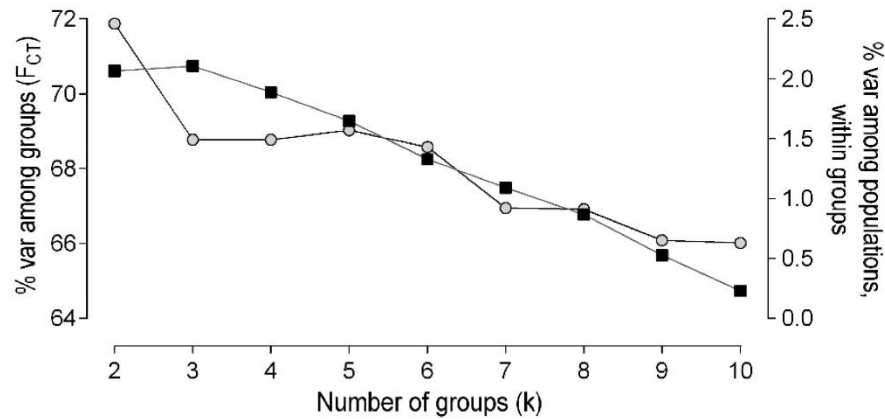
Supplemental Figure 2. Percentage of genetic variability among groups of 18 green turtle foraging grounds - F_{CT} (black squares, left y-axis), and percentage of genetic variability among populations within groups (gray circles, right y-axis), estimated with an analysis of molecular variance (AMOVA).