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Genetic Stock Structure and Differentiation of Green Turtle, *Chelonia mydas*, Rookeries on St. Croix, US Virgin Islands

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Abstract. – Currently, the genetic population structure of only 3 green turtle, *Chelonia mydas,* rookeries is used to categorize the Eastern Caribbean grouping of the South Atlantic distinct population segment. Tissue samples were collected from 66 nesting green turtles on the East End beaches of St. Croix, US Virgin Islands from 2012 to 2015, and we sequenced ~ 800 base pairs of the mitochondrial DNA (mtDNA) control region to characterize the genetic structure and test for differentiation with the adjacent Buck Island rookery. The haplotypes CmA5.1, CmA5.2, and CmA3.1 were identified on the East End beaches. Results of pairwise tests for differentiation were mixed, with frequency-based F_{ST} failing to detect differentiation at the $p < 0.05$ threshold ($F_{ST} = 0.01148$, $p = 0.18503$), and an exact test indicating significant differentiation ($p = 0.02146$). The detection of CmA3.1 and not CmA16.1 within the East End beaches adds to the haplotype diversity previously observed in the Eastern Caribbean region and suggests that genetic diversity has been underestimated in previous studies. Further investigation including mitogenomic markers and nuclear DNA analyses would provide additional clarity as to the population structure in this region.

Key Words. – mtDNA; *Chelonia mydas*; genetic diversity; sea turtle

Sea turtles exhibit natal homing where females return to the regions of their natal beaches (rookeries) to lay their eggs. This natal homing limits the amount of gene flow from rookery to rookery (Avise and Bowen 1994) and delineates the geographic boundaries of breeding populations, which are made up of one or several adjacent rookeries (FitzSimmons 1998; Bjorndal et al. 2006; Formia et al. 2006; Shamblin et al. 2012). The matrilineal mode of inheritance of mitochondrial DNA (mtDNA) means it is well-suited for studying nesting population structure (Bowen et al. 1992). Rookeries with significantly different mtDNA haplotype frequencies are delineated as separate management units (MUs; Moritz 1994). It is vital that the threats to specific MUs be addressed individually. Characteristically high nest-site fidelity would indicate that females from another MU are not likely to repopulate a nesting population that has been extirpated within ecologically relevant timeframes (Bowen et al. 1993). Conservation of all distinct MUs ensures preservation of the greatest genetic diversity within the species (Proietti et al. 2009). Identification of these individual MUs is necessary for strategic planning of conservation efforts and is a priority for US Recovery Plans for sea turtles (National Marine Fisheries Service and US Fish and Wildlife Service 1991).

Green turtles have been classified into 12 distinct population segments (DPSs) under the US Endangered Species Act (ESA 2016). These classifications are based on haplotype distribution as well as known life-history characteristics of breeding populations from around the world (81 FR 20057, 2016). A global phylogeographic analysis based on 386-base-pair (bp) mtDNA sequence data from 127 rookeries identified 12 major regional groupings of evolutionary distinct green turtle, *Chelonia mydas,* MUs (Jensen et al. 2019). These groupings generally correspond to the 12 DPSs described in Seminoff et al. (2015). The Eastern Caribbean grouping, which is classified as part of the South Atlantic DPS, included only 3 rookeries for which data are available—Buck Island, St. Croix (US Virgin Islands [USVI]), Aves Island (Venezuela), and Suriname (Shamblin et al. 2012; Jensen et al. 2019)—despite widespread nesting across the region (Seminoff et al. 2015). Furthermore, the USVI data set was based primarily on green turtle samples from the Buck Island Reef National Monument rookery described by

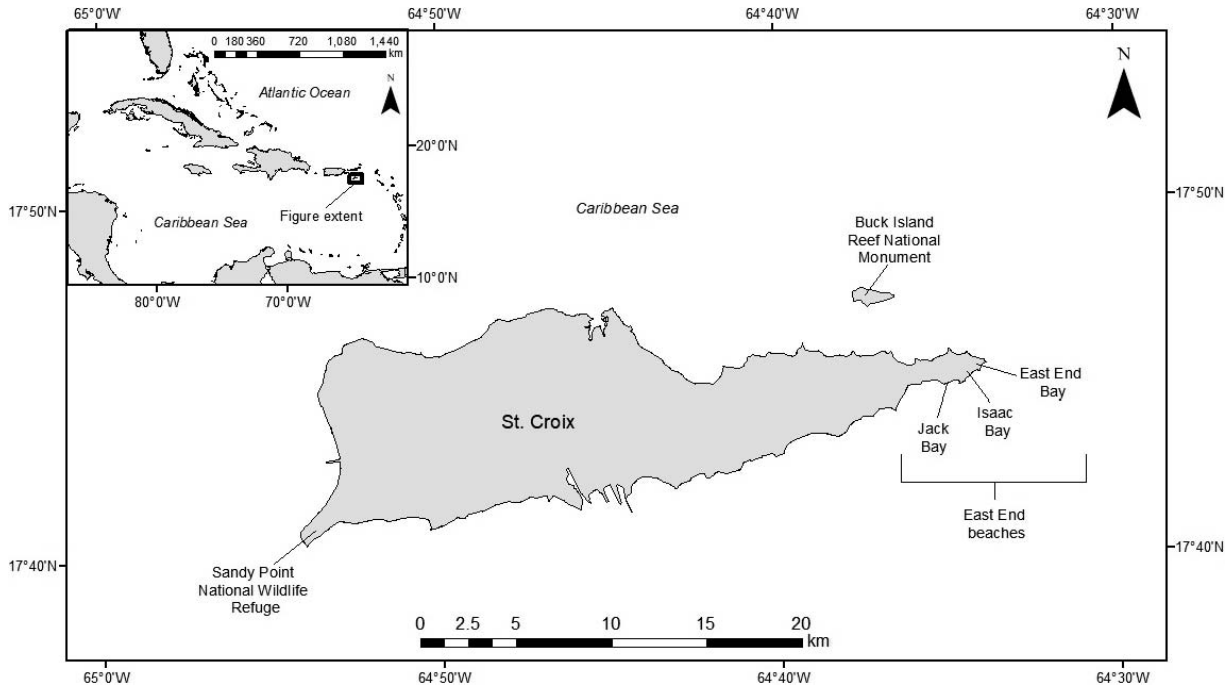

Figure 1. Map identifying Buck Island Reef National Monument, Sandy Point National Wildlife Refuge, and the East End beaches, which are adjacent to East End, Isaac, and Jack Bay in St. Croix, USVI, where green turtle rookeries were studied.

Shamblin et al. (2012). Further research is therefore needed to provide additional data within this area.

Recent surveys have identified significant nesting activity on the East End beaches of St. Croix (USVI), including adjacent beaches within Jack, Isaac, and East End bays with increased green turtle nesting recorded since 2007 (Harvey 2008; E.A. Schultz, unpubl. data, 2016; Fig. 1). This East End rookery is thought to be genetically linked to the rookery on Buck Island because of their close proximity. Shamblin et al. (2012) hypothesized that the Buck Island rookery may be part of a larger USVI genetic stock, and they suggested that further genetic analyses were warranted to clarify the connection between these rookeries. Hill et al. (2018) found that the hawksbill turtle, *Eretmochelys imbricata,* rookery on Buck Island was genetically distinct from the hawksbill rookery on the Sandy Point National Wildlife Refuge, on the west end of St. Croix, USVI, which are only separated by 30 km. These findings give support to the theory that geographic distance is not necessarily a predictive factor for genetic connectivity, as also noted by FitzSimmons and Limpus (2014) and Shamblin et al. (2015). However, Jensen et al. (2019) notes that green turtle rookeries in close proximity (< 500 km) tend to show no genetic differentiation based on their analysis of 127 rookeries globally. To date, no research has been conducted to investigate the connectivity among the relatively large green nesting rookeries (Buck Island and the East End beaches) in St. Croix, which are only separated by 10 km (Fig. 1). Both of these rookeries have > 100 green turtles crawls every year, making them among the largest green sea turtle rookeries in the Greater Antilles region (Dow et al. 2007). The East End beaches average 200–300 green turtle nests/yr (E.A. Schultz, unpubl. data, 2016). The lack of fine-scale genetic structure evaluation of these rookeries constitutes a gap in data necessary for management of this regional nesting population.

Here, we conduct extensive sampling of green turtles nesting on the St. Croix East End beaches in order to characterize the mtDNA diversity. We then compare this data with published findings for the Buck Island rookery (Shamblin et al. 2012) in order to test for fine-scale population structure. We also reexamine population structure within the Eastern Caribbean region by incorporating new data for the East End rookery from our study into the previously published findings.

METHODS

Study Site. — The East End beaches of St. Croix, USVI (17°44′59N, 64°34′21W) are a 600-acre (243-ha) area, which includes approximately 2.0 km of total beach within Jack, Isaac, and East End bays that is located on the southeast corner of the island (Fig. 1). The Nature Conservancy established a sea turtle monitoring program on these beaches in 1994 to document hawksbill and green sea turtle nesting activity through a combination of daytime and nighttime surveys (Harvey 2008). The area is characterized by steep sloped hills with pocket beaches at their base. The East End beaches are located approximately 10 km from the Buck Island Reef National Monument, which is a separate island located to the northeast of the main island of St. Croix (Fig. 1).

Tissue Collection. — The Nature Conservancy collected skin biopsy samples from individual nesting

Table 1. Haplotype frequencies of the green turtle, *Chelonia mydas,* with 817-base-pair (bp) fragment analyses of mtDNA from control region from within the Eastern Caribbean Region. Rookery sites include East End beaches (EEB; present study), Buck Island (BUC), Tortuguero (TRT), Aves (AVE), and Suriname (SUR). CmA3.X and CmA4 counts represent published data based on 490-bp sequences (Shamblin et al. 2012). — = no presence of the haplotype detected from rookery sampled.

Haplotype	EEB	BUC	TRT	AVE	SUR	GenBank
CmA3.X	—	—	393	—	—	
CmA3.1	4	—	2	5	1	JN632497
CmA4	—	—	1	—	—	
CmA5.1	60	44	32	48	55	JN632498
CmA5.2	2	1	—	14	—	JN632499
CmA6.1	—	—	—	—	2	JQ366073
CmA16.1	—	4	—	—	—	JN632500
CmA20.1	—	—	2	—	—	JN632501
CmA21.1	—	—	3	—	—	JN632502
Sample size	66	49	433	67	58	

female green sea turtles on the East End beaches during the nesting season in 2012, 2014, and 2015. Females were sampled using a 6.0-mm biopsy punch during the egg-laying process and skin samples were stored in vials in a saline solution as described by Dutton and Balazs (1995). Each female was then flipper and/or passive integrated transponder (PIT) tagged to avoid duplicate sampling efforts.

Laboratory Analyses. — DNA was isolated from 66 tissue samples using a modified sodium chloride extraction protocol (Miller et al. 1988). An ~ 800-bp fragment of the mitochondrial control region was amplified using primers LCM-15382 (5′ GCT TAA CCC TAA AGC ATT GG 3′) and H950g (5′ GTC TCG GAT TTA GGG GTT TG 3′) and standardized polymerase chain reaction (PCR) procedures (Abreu-Grobois et al. 2006; Dutton et al. 2007). The 25-µl PCR reaction was composed of 1× buffer, 0.8 mM $MgCl_2$, 0.6 mM deoxynucleotide triphosphate (dNTP), 0.3 µM of each primer, 1.25 U *Taq* polymerase (New England BioLabs) and 20–50 ng of template DNA. The PCR was performed using the following profile: initial DNA denaturation at 94°C for 2 min, followed by 30 cycles of 1) DNA denaturation at 94°C for 50 sec, 2) annealing of primers at 56°C for 50 sec, 3) extension of primers at 72°C for 1 min, and 4) extension of primers at 72°C for 5 min. In order to detect contamination, negative controls were included in each PCR. The products were purified and sequenced using procedures similar to Dutton et al. (2014).

Statistical Analysis. — Sequences were edited and aligned using the program Geneious (Kearse et al. 2012) and compared with a reference database to identify haplotypes following the nomenclature for the 817-bp control region fragment on the Archie Carr Center for Sea Turtle Research web site (http://accstr.ufl.edu/resources/mtdna-sequences/). Haplotype frequencies for Buck Island, USVI (BUC); Tortuguero, Costa Rica (TRT); Aves Island, Venezuela (AVE); and Galibi, Suriname (SUR) were utilized from Shamblin et al. (2012). We tested for stock structure by conducting haplotype frequency–based pairwise F_{ST} with 10,000 permutations and exact tests for differentiation with 100,000 steps in Markov chain and 10,000 dememorization steps (Raymond and Rousset 1995) in Arlequin v3.5 (Excoffier and Lischer 2010).

RESULTS

Three haplotypes were identified from the samples collected on the East End beaches of St. Croix (n = 66), which consisted of CmA3.1 (n = 4, 6.0%), CmA5.1 (n = 60, 91.0%), and CmA5.2 (n = 2, 3.0%). Comparison of haplotypes that have been identified based on the 817-bp mtDNA control region fragment within other Caribbean and Atlantic green sea turtle rookeries are listed in Table 1. The F_{ST} results indicated lack of significant differentiation between the Buck Island and East End beach rookeries ($p > 0.1$; Table 2); however, the exact tests results did reveal a significant difference ($p < 0.05$; Table 2).

DISCUSSION

Our study revealed additional genetic diversity within the USVI green turtle population that had not been

Table 2. Pairwise F_{ST} values (above the diagonal) and p-values of exact tests of population differentiation (below the diagonal) among 5 green turtle rookeries based on 817-bp sequence mtDNA haplotypes. Rookery sites include Buck Island (BUC), East End beaches (EEB; present study), Tortuguero (TRT), Aves (AVE), and Suriname (SUR). * = significant at $p < 0.05$.

	BUC	EEB	TRT	AVE	SUR
BUC		0.01148	0.82071*	0.09256*	0.01987
EEB	0.02146*		0.81262*	0.08845*	0.00371
TRT	< 0.0001*	< 0.0001*		0.74275*	0.83414*
AVE	< 0.0001*	0.00520*	< 0.0001*		0.13986*
SUR	0.01649*	0.11882	< 0.0001*	< 0.0001*	

detected in previous studies, which only used data from the Buck Island rookery to represent this region. In addition, our analyses show mixed evidence for genetic differentiation between the East End beaches (EEB) and Buck Island (BUC) rookeries and a need for further mitogenomic analyses. Both of these nesting populations are dominated by the CmA5.1 haplotype (EEB = 91.0%, BUC = 90.0%), but detection of the CmA3.1 haplotype on the East End beaches is novel because that haplotype has not been identified within the Buck Island rookery and adds to the haplotypic diversity previously documented within the USVI. It is unclear whether CmA3 might also be present at Buck Island but was not detected in the samples analyzed by Shamblin et al. (2012). Conversely, we did not detect CmA16, unique to Buck Island, at the East End beaches. Given that the sample size for both studies is fairly representative relative to the small number of nesters, it is possible that this pattern reflects some degree of demographic independence as signaled by the significant differentiation detected with the exact test. Additional sampling of all the beaches in the local region would help establish whether these, and potentially other rarer haplotypes, might be present. Furthermore, the presence of the CmA3 haplotype warrants further phylogenetic analysis to investigate evolutionary history and possible links to the Northwestern Atlantic rookeries. The detection of the CmA16 haplotype solely within the Buck Island rookery also provides additional support for further investigation within this region.

The distance between green turtle nesting beaches is not always a determinant of separate MU classification. Green turtle rookeries in the Rocas Atoll and Fernando de Noronha (off the coast of Brazil) were found to be genetically different based on sequencing of mitochondrial short tandem repeat (mtSTR) haplotypes even though these rookeries are only 150 km apart (Shamblin et al. 2015). However, in the Caribbean there may be more "leakage" (females utilizing other nesting beaches) than first thought by Bowen et al. (1992) because of the close proximity of the islands as noted by nesting female telemetry data from Esteban et al. (2015) and E.A. Schultz et al. (unpubl. data, 2016). Satellite telemetry data from a female nesting on the East End beaches in 2015 revealed this female also nested on Antigua and St. Kitts, nearly 180 km away, during the same nesting season (E.A. Schultz et al., unpubl. data, 2016). Some females in this region seem to exhibit behavioral plasticity in regard to nesting beach selection and may be using beaches in the region interchangeably, although the frequency of occurrence is not well-studied. Bjorndal et al. (2005) also mentions these natal homing "mistakes," but explains that genetic analyses have shown these occurrences to be rare. Data from genetic studies combined with spatial data obtained through traditional tagging (flipper and PIT tags) and satellite telemetry should continue to enhance conservation managers' understanding of the true movements and behavior of turtles from rookeries within the Eastern Caribbean region.

The apparent genetic connectivity identified between the East End beaches and Buck Island may suggest that the East End beaches and Buck Island rookeries should be considered part of a larger USVI breeding population. However, the slight haplotype frequency shift, presence of unique haplotypes, and the low power of the mtDNA marker to detect weak differentiation caution against combining these into one MU. Previous studies illustrate that there are mitogenomic markers outside of the 817-bp control region that have been shown to differentiate the CmA5.1 haplotype, which was identified in both USVI rookeries (Shamblin et al. 2012). Utilizing single nucleotide polymorphisms (SNPs) to further delineate the specific haplotypes within these rookeries that can be broken down into variants would provide more clarity on the connectivity of these populations (Shamblin et al. 2012). Future work should apply these mitogenomic markers on the East End beaches to provide additional insight into the complexity of the adjacent rookeries and better understand the genetic variability within the Eastern Caribbean region. This study could be expanded further to include multilocus nuclear DNA analyses to investigate demographically independent populations within this region. Dutton et al. (2013) found that leatherback, *Dermochelys coriacea,* nesting sites previously classified within the same MU were able to be further differentiated into demographically independent populations (DIPs). The application of mtSTR markers may also be helpful in distinguishing local green rookeries as has been done in the Mediterranean (Bradshaw et al. 2018; Tikochinski et al. 2018), Brazil (Shamblin et al. 2015), and Florida (Shamblin et al. 2020). Additional investigations should also examine the haplotype frequencies of the green sea turtle rookery on the Sandy Point National Wildlife Refuge on St. Croix (as suggested by Hill et al. 2018). It has a very active population for which $>$ 1000 crawls/yr have been observed and is approximately 30 km west of Buck Island and the East End beaches (King et al. 2014; Fig. 1).

The identification of fine-scale differences in rookeries within such a small geographic region could provide evidence that there may be more genetic variability within the green turtle population than understood from current research. The significant differentiation between the East End beaches and Buck Island rookery detected with the exact test provides evidence for considering these as independent MUs. This study is the first step to better understanding the genetic variability present within the green turtle rookeries in St. Croix and additional analyses are needed to fully identify the diversity present in the Eastern Caribbean region.

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