Short Communication

Green turtle (*Chelonia mydas*) genetic diversity at Paranaguá Estuarine Complex feeding grounds in Brazil

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Abstract

Sea turtles are marine reptiles that undertake long migrations through their life, with limited information regarding juvenile stages. Feeding grounds (FGs), where they spend most of their lives, are composed by individuals from different natal origins, known as mixed stock populations. The aim of this study was to assess genetic composition, natal origins and demographic history of juvenile green turtles (*Chelonia mydas*) at the Paranaguá Estuarine Complex (PEC), Brazil, considered a Natural World Heritage site. Tissue samples of stranded animals were collected (n = 60), and 700 bp mitochondrial DNA sequences were generated and compared to shorter sequences from previously published studies. Global exact tests of differentiation revealed significant differences among PEC and the other FGs, except those at the South Atlantic Ocean. Green turtles at PEC present genetic signatures similar to those of nesting females from Ascension Island, Guinea Bissau and Aves Island/Surinam. Population expansion was evidenced to have occurred 20-25 kYA, reinforcing the hypothesis of recovery from Southern Atlantic refugia after the last Glacial Maximum. These results contribute to a better understanding of the dynamics of green turtle populations at a protected area by providing knowledge on the dispersion patterns and reinforcing the importance of the interconnectivity between nesting and foraging populations.

Keywords: *Chelonia mydas*, mtDNA, feeding grounds, mixed stock analysis, connectivity.

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Sea turtles are reptiles that exhibit complex life traits, such as long generation and wide-ranging migrations, inhabiting both different tropical and subtropical regions (Bolten, 2003). As soon as they reach sexual maturity, adults make migrations to their natal beaches for reproduction, termed natal homing (Carr, 1967).

Molecular markers are a useful tool for sea turtle research, as they offer an indirect approach to test behavior, ecology and evolution (Lee, 2008). Population structure studies of these phylopatric species have focused on mitochondrial DNA sequences (mtDNA), as this is a maternally inherited marker. Differences at mtDNA haplotype frequencies among rookeries provide information to link feeding populations with their natal origins (Bowen and Karl, 2007), and may inform of past demographic events (Naro-Maciel *et al.*, 2014).

The methodology known as mixed stock analysis uses Bayesian algorithm to estimate the contributions of different rookeries to a feeding ground (Okuyama and Bolker, 2005; Bolker *et al.*, 2007). The method gives the percentage of each stock as contribution to a foraging aggregation, or mixed stock, and has been proven useful to study the natal origins of green turtles (Bass and Witzell, 2000; Luke *et al.*, 2004; Bass *et al.*, 2006; Bjorndal and Bolten, 2008).

Green sea turtles (*Chelonia mydas* Linnaeus, 1758) are listed as endangered according to IUCN Red List as a result of overexploitation of eggs, adult females at nesting beaches, juveniles and adults at feeding grounds, marine fisheries and pollution; mortality associated with entanglement in marine fisheries and degradation of both nesting

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and foraging habitat also play a role in the decline of many populations (Seminoff, 2004).

The lack of genetic sampling of green turtle feeding grounds precludes the complete understanding of the spatial ecology of these animals (Naro-Maciel *et al.*, 2012 and references therein). The recognition of the importance of an international framework is necessary to assure survival and success of the populations (Bass *et al.*, 2006).

The research at feeding grounds is less developed when compared to rookeries, where females are easily observed (Bowen and Karl, 2007), and much information regarding their life history might be incomplete (Bass and Witzell, 2000). For these reasons, the objectives of this study were (1) to genetically characterize the green turtle population which uses an Atlantic Forest estuarine complex as a feeding ground; (2) to assess and compare the genetic diversity among this study site and previously published data of feeding grounds; (3) to estimate the natal origins of the juvenile green turtles and the connectivity between rookeries and feeding grounds, based on mitochondrial DNA sequences, improving the knowledge about this complex population structure; and (4) to infer demographic events of this population, in order to contribute to understand how climate-related changes may have impacted southern Atlantic green turtle populations.

The sampling was carried out at the Paranaguá Estuarine Complex (PEC) (Figure 1), located at the northern coast of Paraná state in southern Brazil, considered a Natural World Heritage site (UNESCO, 1999). This region has approximately 600 km^2 and includes extensive areas of mangrove forests, sandy beaches and rocky islands (Noernberg *et al.*, 2004). A mosaic of conservation units, including marine and terrestrial units of restricted and sustainable use, is present at the study area. With these different habitats and various food resources, the study site, is a feeding ground for the green turtle (Guebert-Bartholo *et al.*, 2011).

The sampling consisted of monitoring the estuary and sand beaches once every two weeks, from 2005 to 2008. Tissue samples (n = 60) were collected from dead individuals found stranded or floating in the water, and were stored in 100% alcohol. DNA extraction followed the method of Aljanabi and Martinez (1997). The primers LCM15382 and H950 (Abreu-Grobois *et al.*, 2006) were used to amplify approximately 700 base pairs (bp) from the mitochondrial control region using the following PCR protocol: denaturation for 2 min 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. Amplified products were sequenced using BigDye® Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) in an ABI PRISM® 3100 Genetic Analyzer/HITACHI. Sequencing was done by the DNA Sequencing Service (SSDNA) at the University of São Paulo.

Sequences were aligned using BioEdit 5.0.6 (Hall, 1999) and haplotypes were identified according to the Archie Carr Center for Sea Turtle Research. A haplotype network was generated by TCS (Clement *et al.*, 2000), considering the long sequences generated by our primer pairs. The model of nucleotide substitution chosen for the analyses was a Jukes-Cantor following jModelTest (Posada, 2008). The software Arlequin 3.5 (Excoffier *et al.*, 2005) was used to assess haplotype and nucleotide diversity indices. Analyses of molecular variance (AMOVA) and exact tests of differentiation were also performed to study the genetic structure among FGs. To make comparisons with other datasets possible, we shortened our sequences to 480 bp for mixed stock analysis. The other FGs considered in the analyses were: North Carolina (Bass *et al.*, 2006), Florida (Encalada *et al.*, 1996; Bass and Witzell, 2000), Bahamas (Lahanas *et al.*, 1998), Nicaragua (Bass *et al.*, 1998), Barbados (Luke *et al.*, 2004), Almofala and Ubatuba (Naro-Maciel *et al.*, 2007), Rocas Atoll, Fernando de Noronha , Espírito Santo and Bahia (Naro-Maciel *et al.*, 2012), Arvoredo Island (Proietti *et al.*, 2009, 2012), Argentina (Prosdocimi *et al.*, 2012) and Cabo Verde (Monzón-Arguello *et al.*, 2010).

Chi-square tests implemented in CHIRXC software (Zaykin and Pudovkin, 1993) were carried out to test the heterogeneity of this FG among the rookeries from the Atlantic Ocean. Mixed stock analyses were employed to test the origins of the juveniles present at the study area, as well as to assess the connectivity between nesting and feeding grounds of the Atlantic Ocean. The analyses were carried out using two different approaches: "one-to-many" (o2m), which estimates the contributions of different rookeries to one particular feeding ground; and "many-to-many" (m2m), estimating simultaneously the origins and destinations of individuals of multiple source (rookeries) and mixed stocks. The o2m analyses were performed using Bayes software (Pella and Masuda, 2001), and the m2m were done by R programming (R Development Core Team, 2005). Both analyses considered weighted prior probabilities according to number of nesting female at each rookery. The number of nesting females used for the weighted priors was assessed by Naro-Maciel *et al.* (2012). The analyses were run with 60,000 Markov Chain Monte Carlo (MCMC) and burn-ins of 30,000 runs.

The results assessed after Gelman and Rubin shrink factor application indicated convergence of the chains. The possible natal origins used in the analyses were Cuba (Ruíz-Urquíola *et al.*, 2010), Mexico, Surinam, Aves Island and Florida (Encalada *et al.*, 1996), Costa Rica (Bjorndal *et al.*, 2005), Trindade Island (Bjorndal *et al.*, 2006), Ascension Island (Encalada *et al.*, 1996, Formia *et al.*, 2006), São Tomé and Bioko (Formia *et al.*, 2006), Guinea Bissau (Encalada *et al.*, 1996, Bjorndal *et al.*, 2006) and Rocas Atoll (Encalada *et al.*, 1996, Bjorndal *et al.*, 2006).

The historical demographic processes were investigated with Fu's FS (Fu, 1997) and Fu and Li's D* (Fu and Li, 1993) tests, both with 10,000 coalescent simulations in
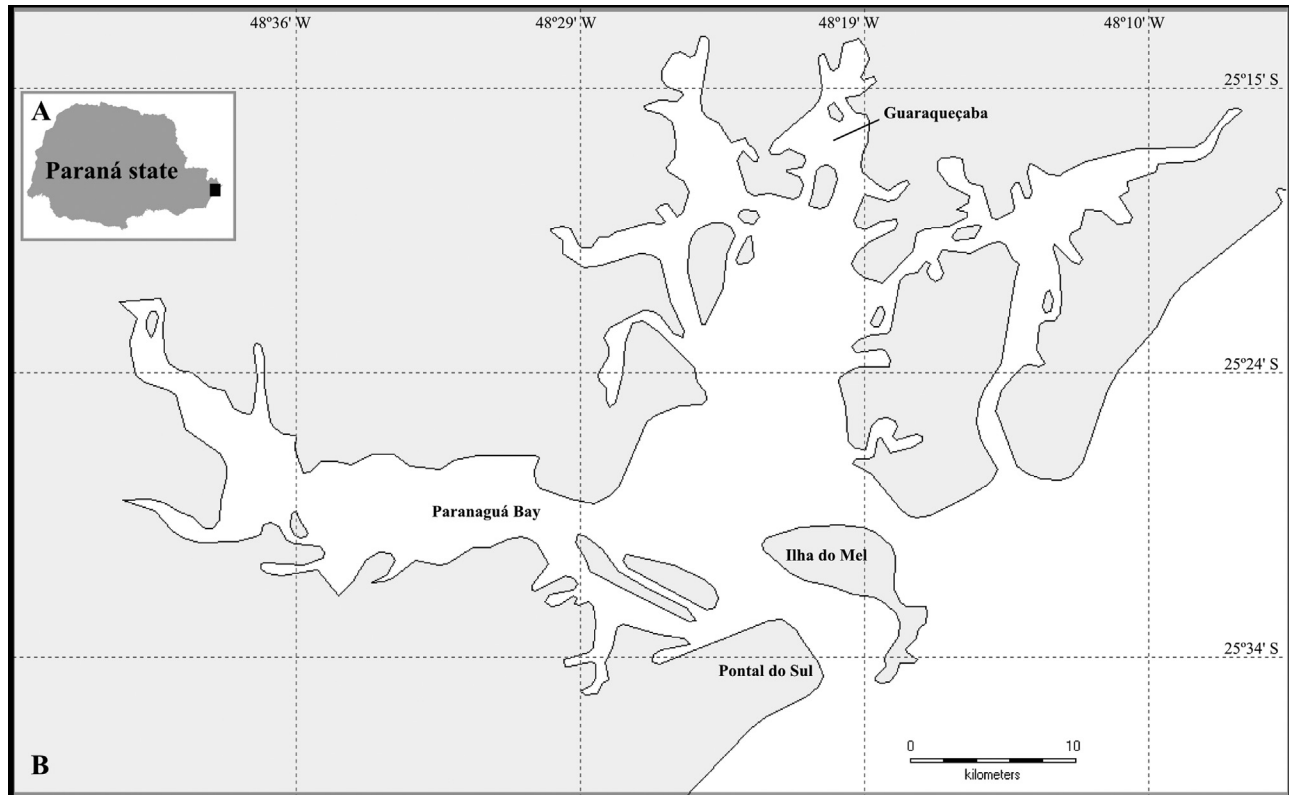
Figure 1 - Map of the study area. A: Map of the Paraná state, in Brazil; the black square indicates the geographic localization of the studied area (detailed in B). B: The Paranaguá Estuarine Complex (PEC), composed by small bays. Names represent main sampling areas of this study (Paranaguá Bay, Pontal do Sul, Ilha do Mel and Guaraqueçaba Bay).

DNASP v5 software (Librado and Rozas, 2009). Timing and extent of demographic changes were inferred by means of the Bayesian skyline plot (BSP) coalescent method implemented in BEAST v1.6.2 software (Drummond *et al.*, 2005). An HKY mutation model without site heterogeneity was used, with a chain length of 100 million iterations. The effective sample size estimator was used to diagnose convergence. A mutation rate of 2.48×10^{-7} / site / generation was considered (Chassin-Noria *et al.*, 2004), and the generation time set at a mean of 40 years.

All the animals sampled were juveniles and subadults. When considering only short sequences from the mtDNA control region, seven haplotypes were found, defined by 11 polymorphic sites: CM-A1, CM-A5, CM-A6, CM-A8, CM-A10, CM-A32 and CM-A46. The most common haplotype was CM-A8 (72%), followed by CM-A5 (13%) and all the other haplotypes were at frequencies less than 5%. For the long sequences, nine haplotypes were recorded (and 14 polymorphic sites), with a subdivision of CM-A8 haplotype in three: CM-A8.1, CM-A8.2 and CM-A8.3. Haplotype diversities (h) for short and long sequences were, respectively, 0.424 ± 0.076 and 0.473 ± 0.075. Nucleotide diversity (π) was 0.001 ± 0.001 for short sequences and 0.002 ± 0.001 for the long sequences.

Global exact tests of differentiation and AMOVA revealed that PEC is significantly distinct from all the other Atlantic FGs (exact p < 0.001, p < 0.001, respectively). In pairwise comparisons, PEC presented no significant difference from Ubatuba (exact p = 0.643 ± 0.003), Arvoredo (exact p = 0.193 ± 0.003) and Argentina (exact p = 0.216 ± 0.003).

Chi-square tests were implemented to test for heterogeneity between the study site and rookeries from Atlantic Ocean. PEC is significantly different from all other rookeries (χ^2 = 4710.53, p < 0.001). The "one-to-many" mixed stock analyses revealed Ascension Island, followed by Aves Island/Surinam and Guinea Bissau as main contributors to PEC. The "many-to-many" analysis indicated Ascension Island as the main contributor to the PEC, followed by Guinea Bissau and Aves Island/Surinam (Table 1).

Long sequence (700 bp) analyses improved the resolution of control region mtDNA haplotypes at the study area, as also shown for other sea turtle studies (Vargas *et al.*, 2008; Shamblin *et al.*, 2012; Dutton *et al.*, 2013). It is worthy of note that the haplotype (CM-A8) revealed a "split" in three (CM-A8.1, CM-A8.2 and CM-A8.3), suggesting that these polymorphisms may be more informative at the resolution of population structure at a regional scale,

Table 1 - "One-to-many" and "many-to-many" mean contributions to PEC, and the upper and lower bounds of the 95% confidence interval.

Stock	One-to-many mean	2.5%	97.5%	Many-to-many mean	2.5%	97.5%
Mexico	0.0061	0.0000	0.0464	0.0226	0.0006	0.0694
Costa Rica	0.0119	0.0001	0.0520	0.0178	0.0004	0.0641
Florida	0.0031	0.0000	0.0321	0.0199	0.0001	0.0714
Aves/Surinam	**0.1363**	0.0603	0.2354	**0.0998**	0.0391	0.1917
Rocas Atoll	0.0009	0.0000	0.0001	0.0078	0.0003	0.0283
Trindade Island	0.0049	0.0000	0.0641	0.0439	0.0019	0.1469
Ascension Island	**0.7423**	0.3049	0.9140	**0.3950**	0.1185	0.6943
Guinea Bissau	**0.0892**	0.0000	0.5257	**0.3508**	0.0913	0.6369
São Tomé	0.0004	0.0000	0.0000	0.0061	0.0002	0.0219
Bioko	0.0048	0.0000	0.0296	0.0284	0.0008	0.1118
Cuba	0.0002	0.0000	0.0005	0.0080	0.0002	0.0254

as shown by Shamblin *et al.* (2012) for green turtle at southern Greater Caribbean rookeries.

Haplotype diversity found at PEC presented a very typical genetic profile with respect to other South Atlantic FGs (Naro-Maciel *et al.*, 2007, 2012; Proietti *et al.*, 2009, 2012; Prosdocimi *et al.*, 2012), differing from the cluster which comprises Caribbean and North Atlantic FGs (Figure 2) (Encalada *et al.*, 1996; Lahanas *et al.*, 1998; Bass and Witzell, 2000; Luke *et al.*, 2004; Bass *et al.*, 2006).

The m2m analysis had wider confidence intervals compared to the o2m. This may explain the difference of mean contributions seen in the two analyses: Ascension Island was considered the main contribution to this study site, although the values were different, the same was the case for Aves/Surinam and Guinea Bissau. Since the current available analyses for sea turtle natal origins present wide confidence intervals, these results should be treated as a qualitative (and not quantitative) estimator, as already suggested by Bowen and Karl (2007).

There are several factors which may be influencing the genetic composition of a feeding ground, such as number of nesting females, geographic distance and/or ocean currents (Lahanas *et al.*, 1998; Bass and Witzell, 2000; Luke *et al.*, 2004). The incorporation of ecological parameters can refine the analyses, yielding more consistent results to the biological reality of the species. As reported so far, Ascension Island, where the number of nesting female in each reproductive season is around 3000 (Seminoff, 2004), is considered the main contributor to all South Atlantic feeding grounds (Naro-Maciel *et al.*, 2007, 2012; Proietti *et al.*, 2009, 2012; Prosdocimi *et al.*, 2012). Furthermore, Ascension Island might be favored by the presence of ocean currents that contribute to the movement of individuals to southwestern Atlantic sites. Trindade Island, on the other hand, is not on the path of some currents that are favorable to green turtle movements towards the South American coast (Prosdocimi *et al.*, 2012), which may explain the low contribution from Trindade Island to PEC feeding grounds,

despite their proximity (2026 km) and source population size (N = 900).

The PEC population showed a significant increase in effective size, with both Fu's FS and Fu's Li tests showing significant departure from neutrality, and BSP showing a five-fold increase since 20-25 kYA. This expansion is interpreted as a population recovery after the Wisconsin Glacial period, when the southern Atlantic provided refugia and thereafter, a source for recolonization of the Atlantic (Naro-Maciel *et al.*, 2014).

The Southwestern Atlantic (ASO) Sea Turtle Network is a network of experts on sea turtles of the South Atlantic that aims to provide and standardize the scientific information available on the biology, conservation and rehabilitation of sea turtles that visit Brazil, Uruguay and Argentina, in order to improve management practices, research, and the support for conservation procedures for these animals. The results obtained in the Paranaguá Estuarine Complex contribute to fill the gaps in our knowledge and, together with the results obtained by other groups in ASO, can guide management decisions. Green turtles look for sheltered habitats to forage, and the occurrence of juveniles highlights the importance of PEC as a developmental habitat in the southwestern Atlantic Ocean (Guebert-Bartholo *et al.*, 2011).

The results of this study provide genetic evidence to support the existence of a highly diverse population, recovering after the Last Glacial Maximum, composed by individuals recruited from multiple nesting beaches of Atlantic Ocean, feeding in the Paranaguá Estuarine Complex. This study emphasizes the importance of characterizing the migratory connectivity between nesting and foraging aggregations, adding a previous unsampled feeding ground to the knowledge on green turtle populations of the Atlantic Ocean. The juvenile phase is the most difficult to understand, and studies concerning this group are of critical importance. Comprehending this step is necessary to create mitigation measures for green turtle protection, including
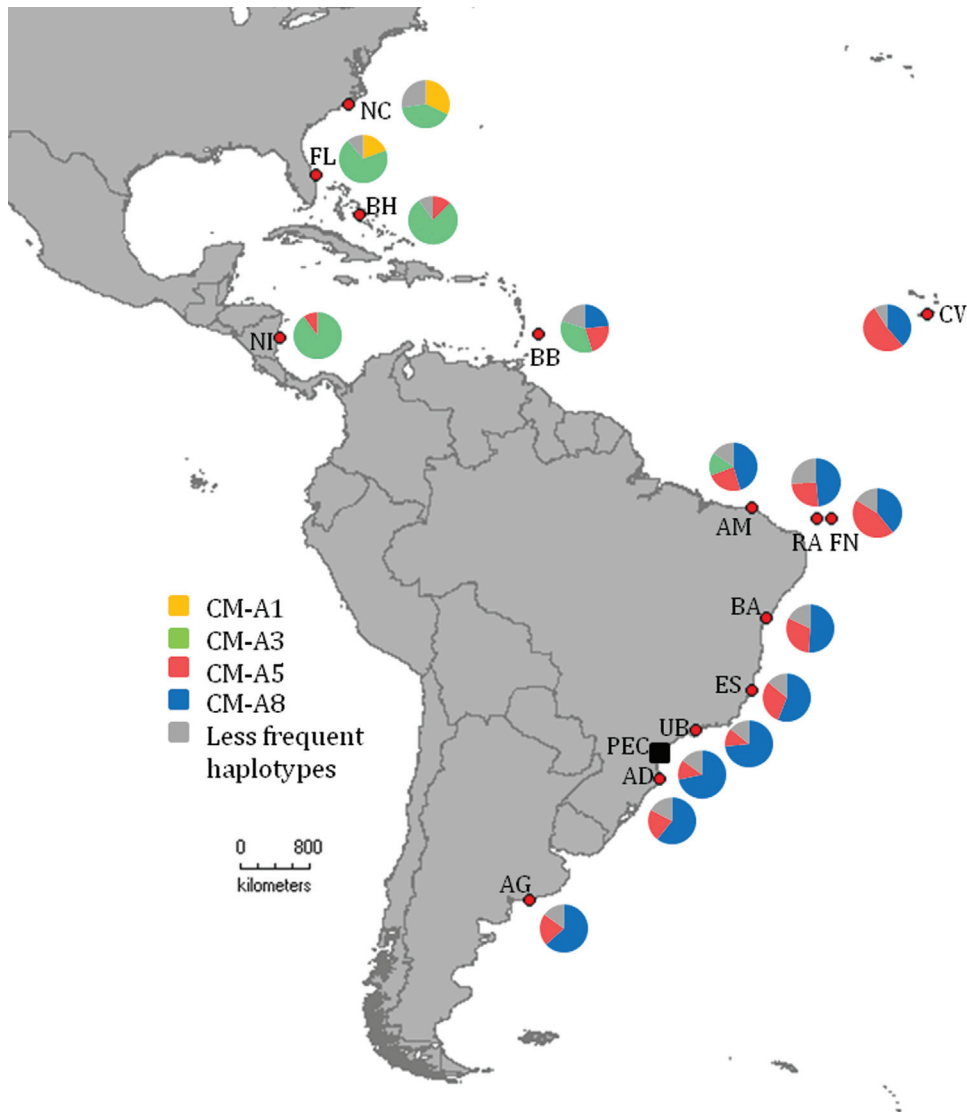
Figure 2 - Genetic composition of Atlantic Ocean FGs. Only the most frequent haplotypes at each FG are represented; the least frequent haplotypes are not identified. The black square represents this study site (PEC) and red circles are for the FGs. Abbreviations are as follow: NC (North Carolina), FL (Florida), BH (Bahamas), NI (Nicaragua), BB (Barbados), CV (Cabo Verde), AM (Almofala), UB (Ubatuba), RA (Rocas Atoll), FN (Fernando de Noronha), ES (Espírito Santo), BA (Bahia), AD (Arvoredo Island) and AG (Argentina).

the identification of priority habitats along the green turtles distribution areas.

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