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RESEARCH ARTICLE

Mixed-stock analysis in green turtles *Chelonia mydas*: mtDNA decipher current connections among west Atlantic populations

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ABSTRACT

The green turtle *Chelonia mydas* undertakes wide-ranging migrations between feeding and nesting sites, resulting in mixing and isolation of genetic stocks. We used mtDNA control region to characterize the genetic composition, population structure, and natal origins of *C. mydas* in the West Atlantic Ocean, at one feeding ground (State of Rio de Janeiro, Brazil), and three Caribbean nesting grounds (French Guiana, Guadeloupe, and Suriname). The feeding ground presented considerable frequency of common haplotypes from the South Atlantic, whereas the nesting sites presented a major contribution of the most common haplotype from the Caribbean. MSA revealed multiple origins of individuals at the feeding ground, notably from Ascension Island, Guinea Bissau, and French Guiana. This study enables a better understanding of the dispersion patterns and highlights the importance of connecting both nesting and feeding areas. Effective conservation initiatives need to encompass these ecologically and geographically distinct sites as well as those corridors connecting them.

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Chelonia, control region, feeding areas, gene flows, MSA, nesting areas

Introduction

Sea turtles are highly migratory animals, spending part of their life cycle in feeding aggregations, which host individuals from different nesting sites separated by long distances (Okuyama & Bolker, 2005). Marine turtles spend most of their lives in marine or estuarine habitats, and the only use of terrestrial habitat is during nesting (Bowen & Karl, 2007; Musick & Limpus, 1997). Relying on the development of telemetry tools and biologgers, studies on the migration of marine animals provided valuable information on their ecology and revealed a remarkable plasticity in their behavior to reduce energy expenditure, when confronted with dynamic environmental conditions during migration (Baudouin et al., 2015; Chambault et al., 2015). Nevertheless, this technology is generally expensive and logistically challenging, given that the marine habitat covers about 70% of the earth's surface. This limits direct observation, capture, and, therefore, monitoring of the species (Waples, 1998). In addition to this spatial challenge, the temporal scale has also to be considered, as these animals present a long generation time, complicating population dynamics approaches. For these reasons, molecular data have long been used to provide information regarding ecology, behavior, evolution, and conservation issues (Bowen & Karl, 1997; Lee, 2008; Molfetti et al., 2013; Naro-Maciel et al., 2007; Plot et al., 2012; Waples, 1998). Most studies on sea turtles use mitochondrial DNA control region (mtDNA) (Bowen & Karl, 2007). These mtDNA genotypes can be used to estimate matrilineal histories of individuals and populations (Avise, 2007, 2009) and to investigate demographic histories (Molfetti et al., 2013; Plot et al., 2012) and geographic structure of populations (Molfetti et al., 2013).

The ability to distinguish nesting aggregations based on haplotype frequencies has clarified dispersal patterns of hatchlings and juveniles (Bass, 1999; Bjorndal et al., 2006; Bowen & Karl, 2007). Feeding grounds are visited by individuals from different natal origins, which, therefore, constitute mixed stocks (Bowen & Karl, 2007). Mixed Stock Analysis thus enables investigating the contribution of each source (nesting site) to a feeding ground, based on the significant differences of haplotype frequencies (Bolker et al., 2003, 2007; Bowen & Karl, 2007; Okuyama & Bolker, 2005). This analysis has proved to be useful for multiple migratory vertebrates, such as salmonids (Grant et al., 1980), whales (Baker et al., 2000; Lukoschek et al., 2009), and sea turtles (Naro-Maciel et al., 2007, 2012; Proietti et al., 2012; Prosdocimi et al., 2012, 2014; Vargas et al., 2008; Vilaça et al., 2013).

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Supplemental data for this article can be accessed here.

For sea turtles, in particular, genetic analysis has been used to map their migrations, and to establish which variables may be most predictive to the utilization of a feeding ground, such as the geographic distance between the sites, number of nesting females, roles of oceanic variables, and importance of historical climatic changes (Bass & Witzell, 2000; Lahanas et al., 1998; Luke et al., 2004; Naro-Maciel et al., 2007; Naro-Maciel et al., 2012). Currently, it is believed that these variables and likely other unknown ecological or geographical variables might act synergistically in determining the aggregation of individuals on the foraging areas. Thus, integrated data analyses including genetic, mark-recapture, at-sea telemetry, and oceanic modeling provide a better understanding of the migration patterns involved (Naro-Maciel et al., 2007).

The distribution of the green turtle is determined by the habitat required for foraging (Balazs, 1980). The species occurs in tropical and subtropical waters. Its distribution within such narrow latitudes is probably explained by the advance of continental ice sheets which led to drop in sea level during glaciations, reducing suitable foraging, and nesting habitats (Encalada et al., 1996). The dietary requirements of the green turtles change during its life as it is mainly omnivorous during its first life stage and gradually shifts to a mainly herbivorous diet in adulthood, constituting feeding groups in coastal waters (Bjorndal, 1997; Ernst et al., 1994). The green turtle is classified as ''endangered'' according to IUCN Red List (IUCN, 2014). The main cause of the population decline is the impact of human activities during all stages of its life (Seminoff, 2004), i.e., bycatch and marine pollution (Casale, 2011; Donlan et al., 2010; Lewison et al., 2004; Oravetz, 1999; Seminoff, 2004).

In order to preserve the green turtle populations, conservation strategies need to be implemented at the relevant geographic and population scale. The concept of Regional Management Units (RMU) (Wallace et al., 2010) of migratory animals relies on the understanding of the complex life history characteristics of the species, and the evaluation of each life stage, in order to understand how population biology has delineated genetic structure over time, in order to make future projections (Bowen & Karl, 1997, 2007; Bowen et al., 2005; Reece et al., 2005). Thus, the objectives of this study were to provide new data to improve the long-term conservation of green turtles, and more widely to contribute to the identifications of Evolutionary Significant Unit/Management Unit (ESU/MU, sensu, Moritz, 1994). More specifically, we aimed (1) to evaluate the genetic diversity of green turtles in one feeding ground and three rookeries in the West Atlantic Ocean; (2) to infer the natal origins of individuals present at the feeding ground; (3) to evaluate contemporary and historical gene flow between the southern feeding ground and the northern nesting sites; and (4) to evaluate, at an unprecedented scale, the structure and spatial connectivity of Atlantic populations, using previously released 2382 sequences together with our dataset of 304 mtDNA sequences. Such data provide relevant information for species conservation, such as identifying key areas serving as refuges to conserve genetic diversity, and others serving as corridors allowing the maintenance of connectivity between nesting and feeding areas.

Material and methods

Field sampling

Tissue samples ($n = 304$) were collected from one feeding ground and three nesting aggregations in the West Atlantic. The feeding ground that has been sampled is on the coast of São Francisco de Itabapoana (SFI), state of Rio de Janeiro, Brazil. Juvenile green turtles' tissues ($n = 190$, carapace length = 37.5 cm ± 8.5) were sampled from live and stranded animals along 36 km of coast from June 2011 to May 2012.

Tissue biopsies were also collected from adult females during the nesting season between 2011 and 2012, in three rookeries: (i) Awala-Yalimapo beach and Cayenne beach, French Guiana (FG, $n = 46$), (ii) Guadeloupe (GD, $n = 36$), and (iii) Babusanti beach, Suriname (SU, $n = 32$). The collected samples were stored at 99% ethanol.

Laboratory procedures

About 20 mg of skin or liver tissue were digested with 15 μL proteinase K (10 mg/mL) and 400 μL lysis buffer at 65 °C overnight (Sambrook et al., 2001). Genomic DNA was extracted following either salt (Aljanabi & Martinez, 1997) or phenol–chloroform protocols (Sambrook et al., 1989). Approximately 780 bp of the mitochondrial control region was amplified using the primers LCM15382 and H950 (Abreu-Grobois et al., 2006). PCR reaction consisted of 20 ng DNA, 1× *Taq* buffer with $(NH_4)SO_4$ (Thermo Scientific, Waltham, MA), 2.5 mM $MgCl_2$ (Thermo Scientific, Waltham, MA), 400 μM of dNTP, 1 U *Taq* DNA polymerase (Thermo Scientific, Waltham, MA), and 0.4 μM of each primer in a 25 μL mix. The amplification profile comprised 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 57 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. Samples from Brazil were sequenced in ABI PRISM® 3100 GeneticAnalyzer (Hitachi, Tokyo, Japan) at the University of São Paulo, whereas other PCR products were sent to Beckman Coulter Genomics (Takeley, UK).

Data analyses

The sequences were assembled, edited, and aligned with MEGA 5 (Tamura et al., 2011), and then compared with those published in the Archie Carr Center for Sea Turtle Research (http://accstr.ufl.edu/resources/mtdna-sequences/) and GenBank (http://ncbi.nlm.nih.gov/) databases, including unpublished sequences and a representative set of published data from main nesting and feeding sites (Figure 1 and Tables 1a and b).

Population structure and genetic diversity

The sequences were trimmed to 490 bp and analyzed for haplotype (h) and nucleotide (π) diversities with Arlequin 3.5 (Excoffier et al., 2005). Exact tests of differentiation (Raymond & Russet, 1995) and analysis of molecular variance (AMOVA) were also performed in Arlequin 3.5 applying a total of 100 000 steps of Markov chains and a burn-in of 50 000 steps, in order to evaluate the genetic structure among the geographic units

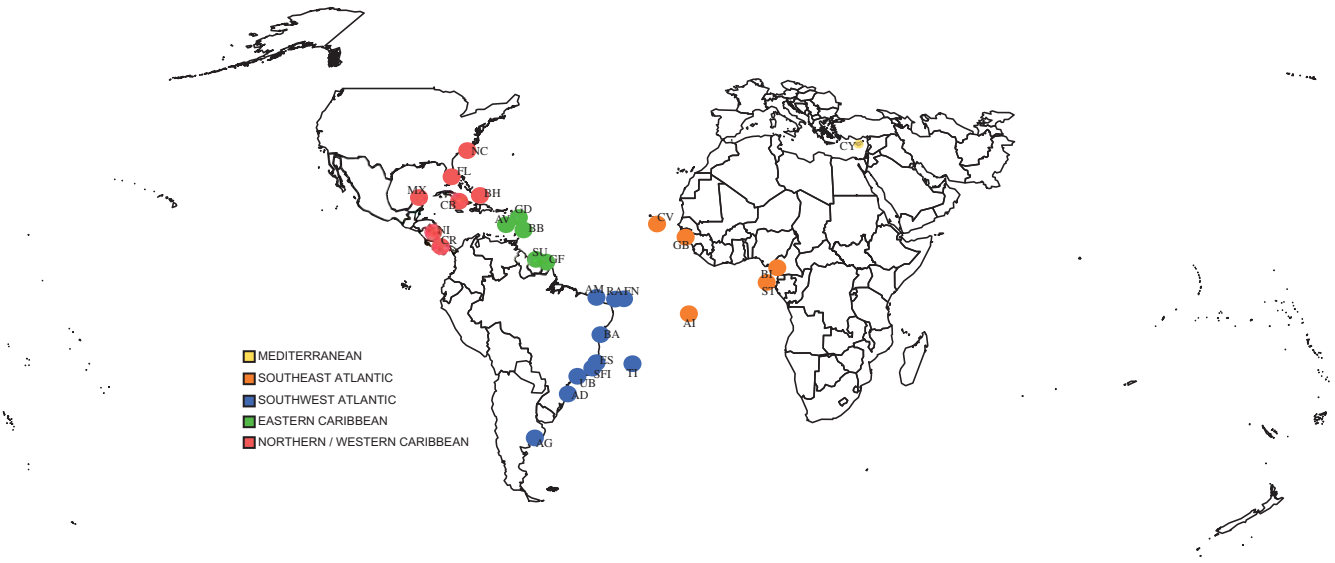

Figure 1. Geographic localization of the feeding grounds and rookeries analyzed in this study, assigned into macro-regions. Abbreviations follow Tables 1 and 2.

Table 1. Feeding grounds considered in this study, haplotypes, and the respective references (superscript numbers).

Haplotype	AM[a]	RA[b]	FN[b]	BA[b]	ES[b]	SFI[c]	UB[a]	AD[d]	AG[e]	CV[f]	BB[g]	NI[h]	BH[i]	FL[j]	NC[k]
CM-A1		1	2			1				1	7		2	12	34
CM-A2														1	2
CM-A3	18	8	4		2	1	2	1		2	21	54	62	43	43
CM-A5	28	26	52	14	47	38	14	25	20	23	13	6	10	3	5
CM-A6	3	4	3	2	7	3		2	2	1					
CM-A8	53	49	46	23	88	130	83	70	59	17	14		1		7
CM-A9	3	9		3	7	4	4	5	5		1				
CM-A10	4	3	4		3	2	3	2	1		2				
CM-A15															1
CM-A16	1														2
CM-A17			1								1				
CM-A18														2	3
CM-A19													1		
CM-A20													1		
CM-A21	1												3		
CM-A22											1			1	2
CM-A23			1	2	1	1		3							
CM-A24	1		1	1	1	3	2	3	1						
CM-A26															2
CM-A27															2
CM-A28															3
CM-A32	1		1		1	2	2	1	2						
CM-A39								1	1						
CM-A42	2		1			3		1	2						
CM-A44	1						1								
CM-A45	1							1							
CM-A46		1					1								
CM-A50						1									
CM-A55							1								
CM-A66			1												
CM-A69						1									
Total	117	101	117	45	157	190	113	115	93	44	60	60	80	62	106

Abbreviation refers as follow: AM, Almofala; RA, Rocas Atoll; FN, Fernando de Noronha; BA, Bahia; ES, Espírito Santo; SFI, São Francisco de Itabapoana; UB, Ubatuba; AD, Arvoredo; AG, Argentina; CV, Cape Verde; BB, Barbados; NI, Nicaragua; BH, Bahamas; FL, Florida; NC, North Carolina.

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

Table 2. Rookeries considered in this study, haplotypes and the respective references (superscript alphabets).

Haplotype	TI[a,b]	RA[a,b]	AI[c,d]	ST[c]	BI[c]	GB[a]	CY[a,e]	FG[f]	SU[g,h]	AV[g]	GD[f]	CR[h]	CB[i]	MX[a]	FL[a]
CM-A1													3	7	11
CM-A2															1
CM-A3										3	1	395	16	5	12
CM-A4												1			
CM-A5				1				43	44	27	35	32		1	
CM-A6			11	1	5				1						
CM-A7									1						
CM-A8	67	36	204	13	45	70		2	1						
CM-A9	19	7	9												
CM-A10		2	5												
CM-A11	1	1													
CM-A12		5													
CM-A13							25								
CM-A14							1								
CM-A15														1	
CM-A16														1	
CM-A17														2	
CM-A18														3	
CM-A20												2			
CM-A21												3			
CM-A22								1							
CM-A23	6		1												
CM-A24	1		7												
CM-A25		1	1												
CM-A27													1		
CM-A28													1		
CM-A32	4	1	1												
CM-A33	1														
CM-A35				1											
CM-A36				1											
CM-A37				1											
CM-A38				2											
CM-A39			1												
CM-A44			1												
CM-A45			1												
CM-A46			2												
CM-A48													5		
CM-A50			1												
CM-A56													1		
CM-A57													1		
Total	99	53	245	20	50	70	26	46	47	30	36	433	28	20	24

Abbreviation refers as follows: TI, Trindade Island; RA, Rocas Atoll; AI, Ascension Island; ST, São Tomé; BI, Bioko; GB, Guinea Bissau; CY, Cyprus; FG, French Guiana; SU, Suriname; AV, Aves Island; GD, Guadeloupe; CR, Costa Rica; CB, Cuba; MX, Mexico; FL, Florida.
[a]Encalada et al. (1996);
[b]Bjorndal et al. (2006);
[c]Formia et al. (2006);
[d]Formia et al. (2007);
[e]Kaska (2000);
[f]This study;
[g]Lahanas et al. (1998);
[h]Bjorndal et al. (2005);
[i]Ruíz-Urquíola et al. (2010).

considered in our study. The haplotype network was generated with a Median Joining strategy, using Network 4.612 software (Bandelt et al., 1999). We assigned geographic macro-regions to the data analyzed (for abbreviations follow Tables 1 and 2): Eastern Caribbean (BB, GD, AV, SU, and FG); Mediterranean (CY); Northern/Western Caribbean (BH, CR, NC, FL, MX, CB, and NI); Southwest Atlantic (AM, FN, RA, BA, ES, SFI, UB, AD, TI, and AG); and Southeast Atlantic (CV, GB, ST, BI, and AI) (see Figure 1). Last, we also investigated the putative cryptic structure with stochastic optimization of Bayesian models as implemented in the computer program BAPS (Corander & Marttinen, 2006). In order to test the number of population clusters, we ran 10 replicates for every level of clusters *k* (*k* ranging from 0 to 20), to identify the most likely *k*. When estimating individual ancestry coefficients, via admixture analysis, we used only clusters that had at least 10 individuals, and we used the recommended number of reference individuals (200) and repeated the admixture analysis 50 times per individual.

Mixed stock analyses

Mixed stock analyses were employed to estimate the natal origins of juveniles green sea turtles sampled in SFI. These analyses follow a Bayesian approach, estimating the contribution of different source populations (rookeries) to a mixed population (Bolker et al., 2007). Foremost, Chi-square tests were performed in CHIRXC software (Zaykin & Pudovkin, 1993) to verify if SFI constituted a mixed stock, testing its heterogeneity compared with the rookeries. Haplotypes found at feeding grounds, which cannot be tracked back to the source

Table 3. Genetic structure among rookeries in Atlantic Ocean.

	TI	RA	AI	ST	BI	GB	CY	FG	SU	AV	GD	CR	CB	MX	FL
TI		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
RA	0.02		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
AI	**0.07**	**0.05**		**0.00**	0.74	0.32	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
ST	**0.09**	**0.07**	**0.10**		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
BI	**0.09**	**0.08**	0.01	**0.06**		**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
GB	**0.10**	**0.09**	**0.01**	**0.17**	**0.10**		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
CY	**0.93**	**0.95**	**0.96**	**0.92**	**0.98**	**1.0**		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
FG	**0.68**	**0.66**	**0.78**	**0.51**	**0.68**	**0.78**	**0.90**		0.49	0.63	1.00	**0.00**	**0.00**	**0.00**	**0.00**
SU	**0.81**	**0.84**	**0.86**	**0.75**	**0.92**	**0.97**	**0.99**	0.03		0.07	0.91	**0.00**	**0.00**	**0.00**	**0.00**
AV	**0.66**	**0.62**	**0.77**	**0.44**	**0.65**	**0.75**	**0.88**	−0.02	0.08		0.32	**0.00**	**0.00**	**0.00**	**0.00**
GD	**0.77**	**0.77**	**0.84**	**0.64**	**0.83**	**0.91**	**0.96**	−0.01	−0.00	0.02		**0.00**	**0.00**	**0.00**	**0.00**
CR	**0.82**	**0.81**	**0.85**	**0.80**	**0.82**	**0.83**	**0.64**	**0.82**	**0.85**	**0.80**	**0.84**		**0.00**	**0.00**	**0.00**
CB	**0.92**	**0.92**	**0.95**	**0.88**	**0.96**	**0.97**	**0.83**	**0.88**	**0.96**	**0.85**	**0.93**	**0.07**		**0.00**	**0.00**
MX	**0.86**	**0.84**	**0.93**	**0.75**	**0.88**	**0.92**	**0.57**	**0.80**	**0.90**	**0.74**	**0.86**	**0.19**	**0.09**		**0.02**
FL	**0.92**	**0.93**	**0.95**	**0.88**	**0.96**	**0.98**	**0.87**	**0.88**	**0.98**	**0.86**	**0.94**	**0.12**	0.01	**0.07**	

Abbreviation names follow Table 2. p Values are above diagonal and population pairwise F_{ST} (Tamura and Nei distance method) are below diagonal. Bold values indicate significant p (<0.05).

population (''orphan'' haplotypes), were excluded from the analyses.

The mixed stock analyses comprised two types of Bayesian approaches: ''many-to-one'', implemented by Bayes software (Pella & Masuda, 2001) and ''many-to-many'', using R programing (R Development Core Team, 2005, mixstock package). At the many-to-one approach, we evaluated the contribution of each nesting population to SFI population with two different strategies: first, we considered that each rookery analyzed would have the same probability to contribute to the mixed stock (equal contribution), by considering the same population size for all rookeries. The second approach consisted of testing if the population size would influence the probability of contributing to the mixed stock (proportional contribution). We divided each rookery population size by the total, so that each rookery is represented as percentage values (e.g. Ascension Island population size = 3709 nesting females, represents 8.6% of a total of 43 124 nesting females accessed), and used it as weighed priori in this latter analysis (Bass et al., 2004; Naro-Maciel et al., 2007).

Many-to-many analyses considered the main rookeries and feeding grounds published (with $N > 20$) for *C. mydas* in the Atlantic Ocean. We evaluated the natal origins in four sets of analyses: (1) all the rookeries and feeding grounds were considered; (2) Cyprus was excluded of the analysis, since preliminary evaluations showed it has a very low contribution to Atlantic feeding grounds (many-to-one analysis and many-to-many results); (3) Guinea Bissau was also excluded as proposed by Godley et al. (2010), which suggested that this nesting aggregation could act as a local population connected to an unsampled feeding ground; and (4) the inclusion of Guinea Bissau and a hypothetic feeding group with the same fixed haplotype (CM-A8, $n = 120$), considering that most sea turtles in Guinea Bissau are restricted to the eastern Atlantic (Godley et al., 2010). Since this rookery presents a fixed haplotype (CM-A8), the hypothetic composition of a local feeding ground would be the same fixed haplotype, following Naro-Maciel et al. (2012).

The number of adult females at each nesting site was provided by Naro-Maciel et al. (2012), except for French Guiana ($n = 6000$) and Guadeloupe ($n = 50$), which were estimated by the field staff of this study. Many-to-one analyses were run with 60 000 Markov Chain Monte Carlo (MCMC) and burn-ins of 30 000 runs, whereas many-to-many analyses took 90 000 MCMC with 30 000 burn-ins. Results were considered when Gelman & Rubin (1992) diagnostic indicated convergence of chains (< 1.2).

Results

Genetic diversity and population structure

We sequenced the mtDNA control region (780 bp) of 304 green turtles in a feeding ground and rookeries in the West Atlantic: SFI (190), FG (46), SU (32), and GD (36). A total of 14 haplotypes, including one undescribed to date (CM-A69, GenBank Accession number KC792574) were identified in the samples (Tables 1 and 2). Haplotype (h) and nucleotide (π) diversities were 0.0556 ± 0.0518 and 0.0012 ± 0.0011 for Guadeloupe; 0.1249 ± 0.0653 and 0.0003 ± 0.0005 for Suriname; 0.1266 ± 0.0655, 0.0027 ± 0.0019 for French Guiana and 0.4929 ± 0.0381 and 0.0014 ± 0.0010 for SFI.

Global exact tests of differentiation unveiled significant structure (exact $p < 0.001$) among all rookeries considered. Pairwise tests (Tamura and Nei distance method) did not reveal significant difference among nesting aggregations found in French Guiana, Guadeloupe, Suriname, and Aves Island. Similarly, these nesting sites did not present significant structuration comparing p-values F_{ST} (Table 3).

For the feeding ground, global exact tests of differentiation revealed that all sites are structured ($p < 0.001$). In pairwise tests, SFI was not significantly different from other southwestern Atlantic feeding grounds: Bahia, Espírito Santo, Ubatuba, Arvoredo, and Argentina (Table 4).

Haplotype network presented a partitioning between two main clades, and BAPS also suggests two clades as the most likely, with 17 haplotypes for the first clade including Northern/Western Caribbean and Mediterranean samples (CM-A1, CM-A2, CM-A3, CM-A4, CM-A13, CM-A14, CM-A15, CM-A16, CM-A17, CM-A18, CM-A22, CM-A26, CM-A27, CM-A28, CM-A48, CM-A56, and CM-A57) and 28 haplotypes for the second clade including Eastern Caribbean, Southwest, and Southeast Atlantic (CM-A5, CM-A6, CM-A7, CM-A8, CM-A9, CM-A10, CM-A11, CM-A12, CM-A20, CM-A21, CMA23, CM-A24, CM-A25, CM-A32, CM-A33, CM-A35, CM-A36, CM-A37, CMA38, CM-A39, CM-A42, CM-A44,

Table 4. Genetic structure among feeding grounds in Atlantic Ocean.

	AM	RA	FN	BA	ES	SFI	UB	AD	AG	CV	BB	NI	BH	FL	NC
AM		0.24	**0.00**	**0.03**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.08	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
RA	0.01		**0.00**	0.15	0.07	**0.00**	**0.00**	**0.02**	**0.02**	**0.03**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
FN	**0.06**	**0.03**		0.07	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.98	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
BA	**0.06**	0.02	0.02		0.58	0.18	**0.01**	0.90	0.38	**0.04**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
ES	**0.08**	**0.03**	**0.02**	−0.01		0.11	**0.00**	0.42	0.31	0.05	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
SFI	**0.09**	**0.04**	**0.09**	0.02	**0.02**		0.29	0.73	0.88	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
UB	**0.08**	**0.04**	**0.12**	**0.07**	**0.05**	0.01		0.23	0.23	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
AD	**0.07**	**0.03**	**0.06**	0.01	0.01	−0.01	0.01		0.93	**0.09**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
AG	**0.08**	**0.03**	**0.07**	0.01	0.01	−0.01	0.01	−0.01		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
CV	**0.06**	**0.05**	−0.01	**0.05**	**0.06**	**0.16**	**0.20**	**0.12**	0.13		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
BB	**0.17**	**0.26**	**0.34**	**0.35**	**0.43**	**0.47**	**0.41**	**0.41**	**0.41**	**0.29**		**0.00**	**0.00**	**0.00**	**0.00**
NI	**0.62**	**0.71**	**0.75**	**0.81**	**0.82**	**0.84**	**0.83**	**0.82**	**0.83**	**0.76**	**0.29**		0.40	**0.00**	**0.00**
BH	**0.51**	**0.60**	**0.66**	**0.68**	**0.73**	**0.76**	**0.73**	**0.72**	**0.73**	**0.63**	**0.18**	0.02		**0.00**	**0.00**
FL	**0.63**	**0.72**	**0.76**	**0.82**	**0.83**	**0.85**	**0.83**	**0.83**	**0.84**	**0.77**	**0.30**	0.02	**0.04**		**0.03**
NC	**0.56**	**0.64**	**0.69**	**0.72**	**0.76**	**0.78**	**0.75**	**0.75**	**0.75**	**0.68**	**0.23**	**0.08**	**0.05**	**0.03**	

Abbreviation names follow Table 1. p Values are above diagonal and population pairwise F_{STs} (Tamura and Nei distance method) are below diagonal. Bold values indicate significant p (<0.05).

CM-A45, CM-A46, CM-A50, CM-A55, CMA66, and CM-A69) (Figure 2). AMOVA confirms those two clades ($F_{ST} = 0.78$; $p < 0.01$), and exact test of differentiation also identifies a structure among the macro-regions ($Pp < 0.01$).

Genetic diversity of clades 1 and 2 was clade 1 (Northern/Western Caribbean and Mediterranean) consisted of 838 individuals, 21 haplotypes, $h = 0.4226 \pm 0.0209$ and $\pi = 0.0043 \pm 0.0027$; while clade 2 (Eastern Caribbean, Southwest, and Southeast Atlantic) consisted of 1848 individuals, 32 haplotypes, $h = 0.6017 \pm 0.0104$ and $\pi = 0.0034 \pm 0.0022$.

When considering macro-regions, some differences were observed: Northern/Western Caribbean, 812 individuals, 19 haplotypes and $h = 0.3860 \pm 0.0211$ and $\pi = 0.0041 \pm 0.0026$; Eastern Caribbean, 219 individuals, 10 haplotypes and $h = 0.4345 \pm 0.0394$ and $\pi = 0.0061 \pm 0.0036$; Southwest Atlantic, 1200 individuals, 26 haplotypes and $h = 0.6026 \pm 0.0135$ and $\pi = 0.0032 \pm 0.0021$; Southeast Atlantic, 429 individuals, 20 haplotypes and $h = 0.3331 \pm 0.0294$ and $\pi = 0.0012 \pm 0.0011$; Mediterranean, 26 individuals, 2 haplotypes and $h = 0.0769 \pm 0.0697$ and $\pi = 0.0002 \pm 0.0003$.

Mixed stock analyses

Chi-square tests indicated SFI as a mixed stock in both overall ($\chi^2 = 1605.18$, $p = 0$) and pairwise tests (data not shown). Many-to-one analyses indicated Ascension Island, Guinea Bissau, and French Guiana as the most important source population to SFI population, considering equal and proportional contributions (Table 5).

Similarly, many-to-many analyses indicated Ascension Island, Guinea Bissau (Southeast Atlantic, mean= 72%), French Guiana, Suriname (Eastern Caribbean, mean= 18%), and Trindade Island (mean= 7%, Southwest Atlantic) as important source populations to SFI population.

Many-to-many analyses recognized French Guiana as an important source population for feeding aggregations in Southwest Atlantic (AM, RA, and ES, mean= 12%; FN, mean= 24%; BA, mean= 15%; AG, mean= 11%; SFI and AD, mean= 10%; UB, mean= 6%); Southeast Atlantic (CV, mean= 38%); Eastern Caribbean (BB, mean= 13%); and Northern/Western Caribbean (BH, mean= 6% and NI, mean= 5%). Suriname presented similar results for foraging sites in Southwest Atlantic (FN, mean= 17%; BA, mean= 16%; ES, mean= 15%; RA, mean= 11%; AD, AG, and AM, mean= 9%; SFI, mean= 8%; UB, mean= 5%), Southeast Atlantic (CV, mean= 13%), and Eastern Caribbean (BB, mean= 7%). Guadeloupe, on the other hand, did not present a relevant contribution to any of the feeding sites analyzed (Table 6 and Figures S1–S15).

Ascension Island is recognized as the main population source for foraging aggregations in Southwest, Southeast Atlantic, and Eastern Caribbean; Guinea Bissau presents the same pattern, and Costa Rica is an important rookery for Eastern Caribbean and Northern/Western Caribbean.

Discussion

Mitochondrial control region of more than 300 green turtles from the Western Atlantic was sequenced to investigate genetic diversity in a feeding ground and three rookeries, to infer natal origins of the feeding ground and to analyze the past and current connectivities between these populations. Information from public databases was added for further analysis in order to gather a dataset of *ca*. 2700 sequences, aiming to gather and provide, at a still unprecedented scale, a more complete view of the populations' structure and the relation between nesting and feeding areas, and more widely to target conservation efforts.

Genetic diversity

CM-A5 haplotype was found in Guadeloupe, French Guiana, and Suriname (the rookeries sampled in this study); it is the most frequent haplotype in the Caribbean region, as previously reported (Bass & Witzell, 2000; Bass et al., 2006; Bjorndal et al., 2005; Lahanas et al., 1994, 1998).

As already observed in other South Atlantic feeding grounds, CM-A8 is the most frequent haplotype in SFI (Naro-Maciel et al., 2007, 2012; Proietti et al., 2009, 2012; Prosdocimi et al., 2012), characteristic of rookeries situated in Ascension Island, Guinea Bissau, and Brazil (Encalada et al., 1996), and which has already been registered in feeding grounds in the Caribbean Atlantic (Lahanas et al., 1998; Luke et al., 2004). The center position of CM-A8 in haplotype networks suggests that it is the closest

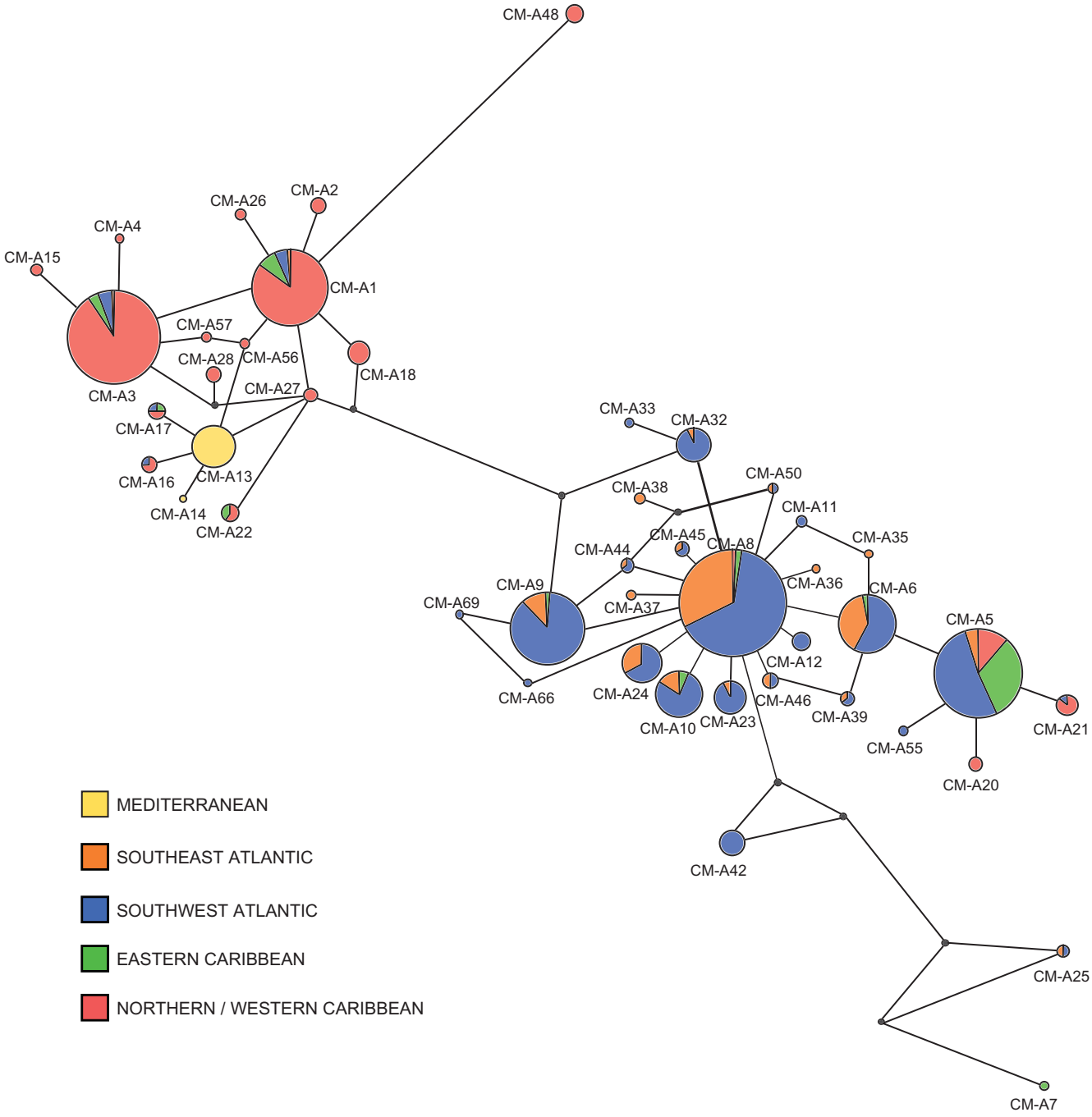

Figure 2. Haplotype network considering five macro-regions. The black dot represents a hypothetical ancestral haplotype which links the current haplotypes. The dot size is proportional to the frequency of each haplotype.

related to the ancestral haplotype (Bjorndal et al., 2006; Encalada et al., 1996). The SFI dataset showed no significant structure among Bahia, Espírito Santo, Ubatuba, Arvoredo, and Argentina. This pattern is consistent with the sharing of juvenile populations foraging across Brazil, Argentina, and Uruguay and supports the hypothesis of the existence of a marine corridor among those areas, as already proposed by Fallabrino et al. (2010).

In highly migratory marine animals, one of the most complex questions is the elucidation of juveniles' movements; usually this stage is difficult to study due to the small body size of individual, which does not allow equipping them with tag, long migrations, and high cost of in-water studies (Bjorndal et al., 2005; Lahanas et al., 1998). The few available studies suggest specific differences in the migration patterns. Mark-recapture data of green turtles in Brazil show movements to north and south, towards feeding aggregates located in Brazil, Central America, Caribbean, and Africa (Marcovaldi et al., 2000). Telemetry and mark-recapture studies on green turtles in Southwest Atlantic also provided information on their dispersal towards north and south, related to seasonal diet preferences (González et al., 2012; López-Mendilaharsu et al., 2006). A recent satellite telemetry study on juvenile loggerhead turtles (*Caretta caretta*) demonstrated that their dispersal is not unidirectional and that they select habitats mainly according to their thermal profile (Mansfield et al., 2014). Similar studies

on the long term might enhance the understanding of the migratory behavior on sea turtles and the impact of migrations on genetic connectivity.

Mixed stock analyses

Mixed stock analysis enables to estimate the contribution of different nesting sites to a feeding ground (Bolker et al., 2007; Okuyama & Bolker, 2005). Chi-square tests provided evidence that SFI is a mixed stock for the green turtle, in overall and pairwise comparisons. The genetic profile of SFI is similar to other South Atlantic feeding grounds (Naro-Maciel et al., 2007, 2012; Proietti et al., 2012; Prosdocimi et al., 2012), with most haplotypes being typical of South Atlantic populations, whereas three of them were mainly described for Northern/Western Caribbean Atlantic populations (CM-A5, CM-A1, and CM-A3) and the last one (CM-A69) was recorded for just our study site (South Atlantic).

Regarding the many-to-many stocks analysis, the results changed depending on the combination of data used, especially in the set 3, when Guinea Bissau was excluded from the analysis (Figures S1–S15). SFI received major contributions from Ascension Island, Guinea Bissau, and French Guiana; as well as minor contributions from Suriname, Aves Island, and Trindade Island. The contribution of Guinea Bissau, i.e. from a nesting site that requires transoceanic migrations, has been discussed and remains not fully solved. Indeed, unlike the prediction of many-to-many MSA (Naro-Maciel et al., 2012), simulations of passive migrations suggested only limited contributions of Guinea Bissau to southern Brazil foraging sites (Putman & Naro-Maciel, 2013). Our results nevertheless reinforce the previous MSA analysis, and together with evidences from telemetry studies (Baudouin et al., 2015; Chambault et al., 2015), using Argos/GPS Fastloc satellite tags to monitor migration after the nesting season of 16 green turtles, support the importance of a coastal corridor between Southwest Atlantic and Caribbean: there are movements, for example, from Guadeloupe and French Guiana nesting sites (''Atlantic, South Caribbean'') in the direction of SFI feeding grounds (''Atlantic, Southwest''). This migration requires to cross the Amazon plume and to cope with the strong counter North Brazil, supposing high energetic costs. A hypothesis is that this strategy could enable the access to highly important feeding resources, as well as facilitate the pre-nesting migration due to favorable currents, saving energy for the highly stressful nesting period in nesting sites of high quality. A similar strategy, with optimization of feeding and nesting areas associated with a reduction of energy costs of migration, could also be considered for transoceanic migrations.

Many-to-many results suggested that South Atlantic and Eastern Caribbean feeding grounds have major contributions from Ascension Island, Guinea Bissau, French Guiana, and Suriname. For Northern/Western Caribbean feeding grounds, Costa Rica is a very important source population, as well as for Caribbean feeding grounds.

Several variables may contribute to influence the connectivity between feeding and nesting sites, such as the geographic distance, number of nesting females, currents, and importance of historical climatic changes (Bass & Witzell, 2000; Lahanas et al., 1998; Luke et al., 2004; Naro-Maciel et al., 2007, 2012). Many-to-many and many-to-one (proportional contribution) analyses were performed considering the number of nesting females at each rookery. The many-to-one analysis, which did not consider this covariate (equal contribution), presented larger confidence intervals when compared with the

Table 5. Many-to-one mean contributions. 2.5% and 97.5% refers to the upper and lower bounds of the 95% confidence interval.

Rookeries	Mean[a]	2.5%[a]	97.5%[a]	Mean[b]	2.5%[b]	97.5%[b]
TI	0.0105	0.0000	0.1002	0.0034	0.0000	0.0486
RA	0.0060	0.0000	0.0667	0.0002	0.0000	0.0000
AI	0.5814	0.2226	0.8060	0.6341	0.2832	0.8154
ST	0.0020	0.0000	0.0206	0.0001	0.0000	0.0000
BI	0.0299	0.0000	0.3167	0.0046	0.0000	0.0041
GB	0.1391	0.0000	0.4882	0.1240	0.0000	0.4734
CY	0.0004	0.0000	0.0041	0.0000	0.0000	0.0000
FG	0.1774	0.0125	0.2786	0.2115	0.0855	0.2887
SU	0.0133	0.0000	0.1436	0.0082	0.0000	0.1097
AV	0.0126	0.0000	0.1311	0.0033	0.0000	0.0441
GD	0.0194	0.0000	0.1758	0.0004	0.0000	0.0000
CR	0.0010	0.0000	0.0101	0.0070	0.0000	0.0253
CB	0.0014	0.0000	0.0139	0.0001	0.0000	0.0002
MX	0.0022	0.0000	0.0178	0.0017	0.0000	0.0158
FL	0.0035	0.0000	0.0214	0.0013	0.0000	0.0139

[a]Equal contribution.
[b]Proportional contribution.
Main contributions are highlighted.

Table 6. Mixed-stock centric mean contributions.

	Feeding grounds														
Rookeries	AM	RA	FN	BA	ES	SFI	UB	AD	AG	CV	BB	NI	BH	FL	NC
TI	0.04	0.10	0.03	0.16	0.08	0.07	0.07	0.13	0.10	0.03	0.03	0.00	0.00	0.01	0.02
RA	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01
AI	0.38	0.38	0.28	0.24	0.38	0.34	0.50	0.43	0.41	0.11	0.13	0.00	0.01	0.01	0.02
ST	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01
BI	0.03	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.02	0.00	0.00	0.01	0.02
GB	0.10	0.09	0.10	0.13	0.17	0.38	0.26	0.15	0.20	0.18	0.08	0.01	0.01	0.01	0.03
CY	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.01
FG	0.12	0.12	0.24	0.15	0.12	0.10	0.06	0.10	0.11	0.38	0.13	0.05	0.06	0.03	0.02
SU	0.09	0.11	0.17	0.16	0.15	0.08	0.05	0.09	0.09	0.13	0.07	0.01	0.02	0.02	0.02
AV	0.05	0.06	0.07	0.06	0.05	0.04	0.04	0.05	0.05	0.05	0.04	0.01	0.01	0.02	0.02
GD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CR	0.16	0.07	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.05	0.31	0.90	0.85	0.65	0.19
CB	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.07
MX	0.02	0.02	0.03	0.02	0.01	0.01	0.01	0.01	0.01	0.03	0.13	0.01	0.03	0.19	0.31
FL	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.03	0.06	0.00	0.01	0.04	0.28

The table presents the mean contribution of each rookery to each feeding ground, after four set of many-to-many analyses. Main contributions are highlighted.

many-to-one which did (Table 5). This result was already expected, since the use of ecological covariates has been demonstrated to be important for this kind of analysis (Lahanas et al., 1998). Despite being useful, there are limitations on the mixed stock analysis, such as rookeries that do not differ from each other in the haplotype composition, the preclusion that all rookeries are sampled and the presence of orphan haplotypes (Bowen & Karl, 2007). Although the large confidence intervals generated as a result of these limitations, this method seems valuable, once it has already described the genetic composition of feeding grounds in different sea turtle species (Bowen & Karl, 2007).

Since our many-to-many analysis relies on a wide integrated dataset partly derived from previously published studies (Tables 1 and 2), it is therefore necessary to improve the genetic sampling for rookeries and feeding grounds worldwide. There is already this type of cooperation with ASO network, specialists from Brazil, Argentina, and Uruguay that cooperate on providing information on the biology and conservation of sea turtles. These countries are already recognized as foraging and migratory corridor for five of the seven species of sea turtles: *Chelonia mydas, Caretta caretta, Dermochelys coriacea, Eretmochelys imbricate,* and *Lepidochelys olivacea* (Fallabrino et al., 2004, 2010; Naro-Maciel et al., 2012).

Conservation issues

The green sea turtle is in danger of extinction, and most regional populations are in decline (Seminoff, 2004). For adequate geographic and ecological scaling, species conservation plans should take into account the genetic structure and demographic history of populations (Lande, 1988). When considering sea turtles, genetic clades may vary according to the gender, age, and bioecological function (e.g. feeding versus breeding) taken into account. The genetic structure of *C. mydas* in the West Atlantic revealed movements of individuals between southern feeding areas and northern nesting rookeries, and reinforces the conservation importance of a regional corridor between the northern Surinamese and French Guianan sites, and Brazil (Baudouin et al., 2015). Furthermore, the presence of an already recognized foraging migratory corridor among south Brazil, Argentina, and Uruguay (Fallabrino et al., 2004, 2010; Naro-Maciel et al., 2007) was also confirmed by our study.

Evolutionarily Significant Units (ESUs) are implemented in order to include sufficient genetic diversity to retain evolutionary potential and address long-term conservation issues; whereas Management Units (MUs) consider functionally independent populations and address short-term conservation targets (Moritz, 1994). A major challenge is to prioritize schemes when multiple population segments (e.g., animals from a feeding ground and/or a nesting site) meet the criteria of a MU and all of them require a specific conservation strategy. The concept of Regional Management Units (RMU) proposes the classification of marine turtles into units of protection, above the regional level of individual breeding populations, with regional entities that can be connected by gene flow. There are 17 RMUs recognized for green turtles (Wallace et al., 2010). Most of the samples analyzed in this study are geographically located in two of these RMUs: the ''Atlantic, South Caribbean'' and ''Atlantic Southwest''. The RMUs present an innovative approach to set units of protection for sea turtles, however, the efficiency of their geographic definitions remains highly dependent on the available data. Our study on the green turtle supplies significant genetic analyses at an unprecedented scale for Western Atlantic, and should be considered in the future to improve the delimitation of the RMUs already recognized.

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Declaration of interest

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