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Population origin and historical demography in hawksbill (*Eretmochelys imbricata*) feeding and nesting aggregates from Brazil

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ABSTRACT

We studied hawksbills from Brazilian feeding aggregates and nesting colonies to ascertain the origin and genealogical relationship of individuals in the largest southern Atlantic remnant population by using sequences of the mitochondrial (mtDNA) control region and five autosomal genes. A phylogeographic analysis of 246 hawksbills showed four distinct mtDNA haplogroups in the feeding grounds, while only one was found in Brazilian rookeries. We found significant differences among nesting sites in Brazil, and among them and other rookeries worldwide. Differences among Brazilian feeding aggregation sites and others around the world were also found. We were able to show that hawksbills from feeding aggregates at the Brazilian islands of Fernando de Noronha and Rocas Atoll were mainly derived from Brazilian and Caribbean rookeries, although some were related to individuals from the eastern Atlantic and Indo-Pacific, indicating large transoceanic migrations for this species. The nuclear data presented no structure and no signal of demographic change. Mixed stock analyses indicated that Brazilian rookeries contribute mostly to Brazilian feeding grounds, and in a smaller proportion to feeding aggregations in the Caribbean and eastern Atlantic. Finally, hybrids found frequently in rookeries of the Bahia State are not present in the feeding grounds, and thus, may display different feeding and migratory behaviors.

1. Introduction

The hawksbill turtle, *Eretmochelys imbricata* (Linnaeus, 1766), is found in tropical areas in the Atlantic and Indo-Pacific regions (IUCN, 2012), associated to coral reefs and shallow environments due to its unique sponge eating habit (Meylan, 1988). Nesting hawksbills can be found in more than 60 countries worldwide, although in low numbers due to the intense exploitation of the carapace scutes (tortoiseshell) that are used to produce expensive luxurious items (Meylan and Donnelly, 1999). Today, there are less than ten rookeries around the world where more than 1000 females are estimated to nest every year (Spotila, 2004). Egg poaching, slaughter of nesting females, habitat destruction, coastal development, and incidental capture by fisheries have also contributed to decrease populations (IUCN/SSC Marine Turtle Specialist Group, 2003).

The species is considered critically endangered by the World Conservation Union (IUCN) and it is listed in the Appendix I of the Convention on International Trade in Endangered Species (CITES). However, non-CITES countries continue to sell and trade tortoiseshell products. Currently, there is an ongoing debate on how the harvest in one country can affect nesting populations located outside its boundaries, which is sustained by the mixed stock analyses of areas subjected to exploration (Bowen et al., 2007; Mortimer et al., 2007). *E. imbricata* is one of the five marine turtle species that can be found in the Brazilian territory, being the fourth in the country in terms of nest abundance (Marcovaldi and Laurent, 1996). It is also considered "critically endangered" in the Brazilian government (IBAMA) official list of endangered species, and protected by a law that covers all life history stages including eggs and hatchlings (Marcovaldi et al., 2011).

The hawksbills share several life history traits with other marine turtles including migratory behavior, nesting site fidelity and natal homing behavior (Bass et al., 1996; Broderick et al., 1994; Carr et al., 1966; Diaz-Fernandez et al., 1999; Miller et al., 1998). During their life cycle, dispersing individuals can migrate over long distances, frequently hundreds and even thousands of kilometers (Horrocks et al., 2001; IUCN/SSC Marine Turtle Specialist Group, 2003; Meylan, 1999; Miller et al., 1998) with some few reports of trans-Atlantic migrations (Bellini et al., 2000; Grossman et al., 2007; Marcovaldi and Filippini, 1991).

The long-distance migratory behavior and long life-span make it difficult to study the life history of marine turtle species and, consequently, it can be hard to design effective conservation strategies

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that need to be planned in several different geographic scales (Bowen and Karl, 2007). The emerging picture indicates that there is no effective conservation when individual countries protect the rookeries in-between their boundaries, but allow harvesting of turtles in feeding aggregates (even thousands of miles away) (Bowen et al., 2007; Bowen et al., 1996; Troeng et al., 2005). Although compiling information about nesting colonies, their genetic distinctiveness and degree of isolation is mandatory for conservation purposes in a regional scale additional knowledge about the demographic links between nesting colonies and feeding aggregates around the world is also essential to design effective management strategies worldwide.

Little is known about juvenile migrations, and the origin and composition of feeding aggregates. Newborns enter the water to start an open ocean interval where their movements seem to be mainly determined by oceanic currents (Carr, 1987; Musick and Limpus, 1997). After a period that can last several years, they migrate to shallow water feeding grounds or developmental habitats (Carr et al., 1966). The composition of turtle aggregates in these developmental habitats is believed to be determined by several factors like the size of regional nesting colonies, oceanic currents and the distance to the contributing nesting colonies, among other factors (Bass et al., 1996; Carr and Meylan, 1980; Engstrom et al., 2002; Lahanas et al., 1998; Luke et al., 2004; Rankin-Baransky et al., 2001; Witham, 1980).

Even though Brazilian rookeries had suffered a long history of exploitation that led to a great decline of turtle numbers and rookery sites (Marcovaldi et al., 1999), the population nesting in Brazil is the largest remnant in the South Atlantic. It is also one of the few sites where more than 1750 nests are registered every year (Marcovaldi et al., 2007). Currently, the effective conservation efforts directed by the Brazilian Project for the Conservation of Sea Turtles (Projeto TAMAR-ICMBio) is reversing the declining trend, thus leading to the growth of the Brazilian nesting population, an important stronghold for the species conservation (Marcovaldi et al., 2007).

Therefore, considering that the majority of data on this species comes from the study of Caribbean and eastern Atlantic populations, the genetic characterization of the relatively large nesting populations and the juvenile aggregates found in the feeding habitats located along the Brazilian coastline can increase significantly the current knowledge on the species migratory patterns along the entire Atlantic basin.

In this study, we describe the analyses of mitochondrial DNA (mtDNA) haplotypes and autosomal variation in the feeding aggregates and the nesting colonies found in Brazil. We studied the phylogeographic patterns that emerged from mtDNA sequences, comparing with data for feeding and nesting aggregates in the Caribbean and eastern Atlantic. We also performed a multiple stock analysis to ascertain the possible origin of juveniles found in the main Brazilian feeding area in order to establish links between this and other populations around the world. One of the main goals is to investigate the connection between these juvenile feeding grounds and rookeries in the Bahia State of Brazil, where previous studies with mtDNA and autosomal sequence data (Lara-Ruiz et al., 2006; Vilaça et al., 2012) indicated an extremely high incidence of hybridization, particularly with loggerheads (*Caretta caretta*). Based on the peculiarity of populations nesting and feeding in the Brazilian territory, some important conservation concerns are envisaged.

2. Materials and methods

Tissue samples (n = 246) were collected by TAMAR-ICMBio field staff between 1999 and 2005 at several nesting localities along the Brazilian coastline and off-shore at feeding areas (Fig. 1). For the nesting population we used samples from the States of Bahia (BA, n = 119, all *E. imbricata* samples used in Lara-Ruiz et al. (2006)), Ceará (CE, n = 2), Rio Grande do Norte (RN, n = 27), and Sergipe (SE, n = 4), which covers all main *E. imbricata* nesting sites found in the Brazilian territory. Samples from juveniles were collected in feeding areas near Fernando de Noronha archipelago (FN, n = 54) and Rocas Atoll (AR, n = 40), in the north eastern coast of Brazil (Fig. 1). The collectors were trained to identify the different species that can be encountered in Brazilian grounds following international standards described in Eckert et al. (1999). For individual identification, all animals were tagged on the front flippers with Inconel tags (National Band and Tag Co. style 681).

Tissue samples were collected using a 6 mm disposable biopsy punch, stored in absolute ethanol and kept at room temperature until their processing in the laboratory. Total DNA was isolated from the samples using the standard phenol–chloroform protocol (Sambrook and Russell, 2001) with modifications introduced at the laboratory (available on-line at http://www.icb.ufmg.br/labs/lbem/protocolos). A 1000 bp fragment including ~800 bp of the mtDNA control region was amplified using primers LCM15382 and H950 (Abreu-Grobois et al., 2006) and a PCR amplification profile of 5 min at 94 °C, followed by 36 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C and a final extension step of 10 min at 72 °C.

PCR products were purified using Polyethylene Glycol 8000 20%–NaCl 2.5 M, and submitted to sequencing reactions using the ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) according to manufacturer instructions. Sequencing reactions were analyzed in an automated MegaBACE 1000 DNA sequencer. At least two independent PCR products from each sample were sequenced using both forward and reverse primers. The chromatograms were base called using Phred 0.020425 (Ewing et al., 1998), and the raw sequences were aligned and edited to produce a high quality consensus sequence for each individual using Phrap 0.990319 (Ewing and Green, 1998) and Consed 19.0 (Gordon et al., 1998).

For further analyses and characterization of polymorphic sites and haplotypes, the consensus sequences for all individuals were aligned using MEGA 4.1 (Kumar et al., 2008). The defined haplotypes start at the first nucleotide after the $tRNA_{Pro}$ and encompass 740 bp of the control region left domain. Haplotypes were named following the same nomenclature for *E. imbricata* samples from Brazil already published (Lara-Ruiz et al., 2006). The sequences were compared with known *E. imbricata* haplotypes (Table 1). All haplotypes found were deposited in GenBank under the accession numbers DQ177335–DQ177341 and JX289882–JX289891.

The relationships among Brazilian *E. imbricata* haplotypes (740 bp) were inferred using a median joining network analysis (Bandelt et al., 1999) implemented in the program Network 4.6 (www.fluxus-engineering.com). In this analysis we excluded haplotypes more related to other species mtDNA, considered here as evidences of hybridization (see Lara-Ruiz et al., 2006).

Estimates of haplotype (h) and nucleotide (π) diversity, as well as Tajima's D and Fu's Fs neutrality tests were generated using Arlequin v3.5 (Excoffier and Lischer, 2010). Analyses of molecular variance (AMOVA) and exact tests of population differentiation were also carried out in Arlequin to evaluate several population divisions and groupings of populations according to geography. The significance of the associated p-values were computed with 10,000 permutations for the AMOVA and population pairwise genetic distances (Φ_{ST}). To evaluate differences between Brazilian populations and other known Caribbean nesting and feeding aggregations, 384 bp mtDNA haplotypes and 740 bp haplotypes, when available (Table 1), were used to compare with short sequence data compiled by Bowen et al. (2007), with additional data from Monzón-Argüello et al. (2010) for Cape Verde, Blumenthal et al. (2009) for Cayman Islands, Monzón-Argüello et al. (2011) for Principe Island, Leroux et al. (2012) for Caribbean longer sequences, Velez-Zuazo et al. (2008) for longer sequences from Puerto Rico, and alterations for haplotype frequencies for the Barbados' rookeries from Browne et al. (2010). Because most sequences used for these comparative analyses were shorter, several of the described haplotypes were binned into one sequence resulting in lower (but comparable) values of haplotype diversity for the Brazilian samples. Data in Bowen et al. (2007) from Venezuela and Brazil were excluded from the analysis because of the small sample

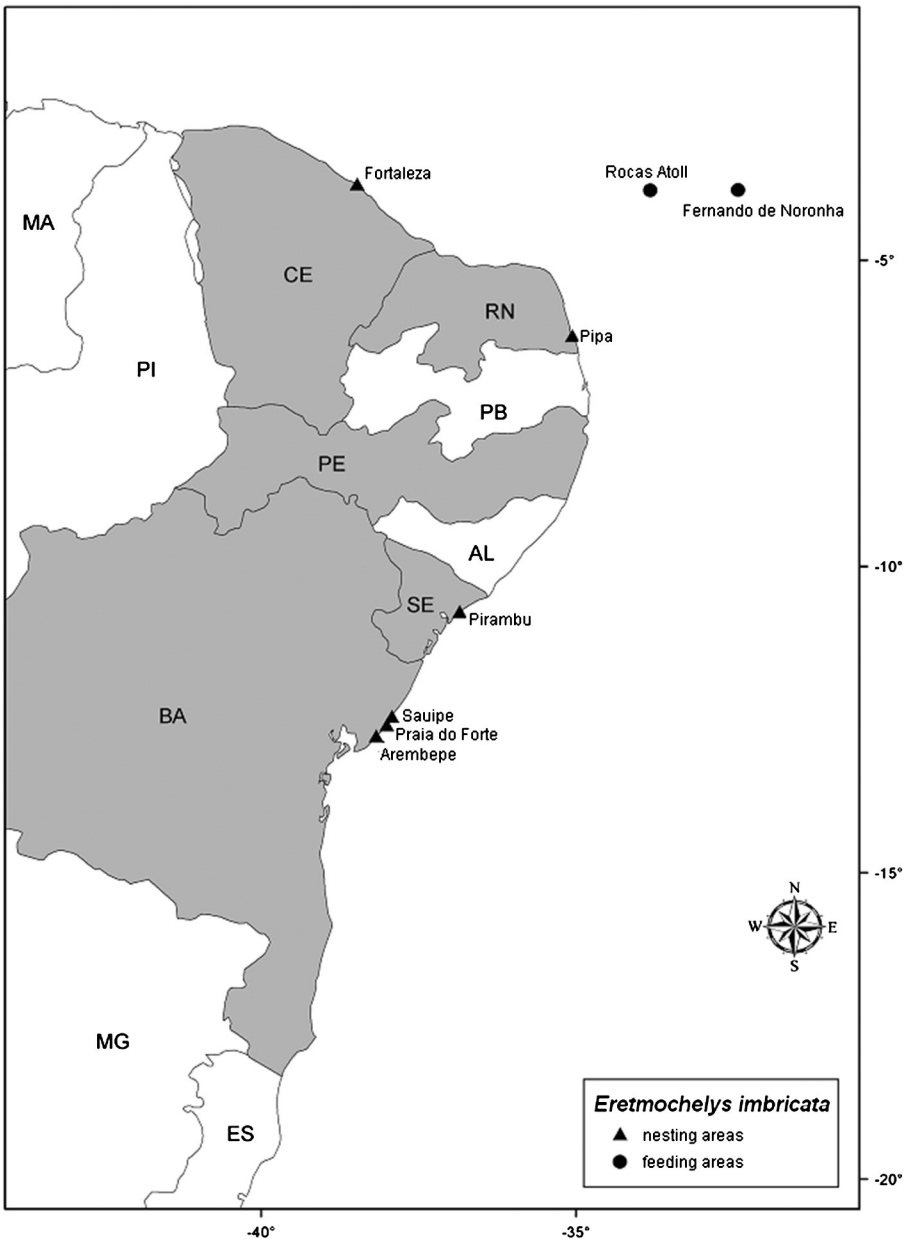

Fig. 1. Location of nesting beaches and feeding areas where *E. imbricata* samples were collected. The samples from Rocas Atoll and Fernando de Noronha constitute the feeding area BR-FEED. The beaches Sauípe, Praia do Forte, and Arembepe corresponds to the rookery BR-BA; Pipa corresponds to BR-RN; Pirambu to BR-SE; and Fortaleza to BR-CE.

sizes, and their data sets for Puerto Rico (two data sets) and Cuba (three data sets) were pooled, and the information regarding the haplotype designated "Cum" was not used since it is identical to the "DR1" haplotype (according to GenBank on September 2011).

To estimate the relative contributions of different nesting colonies to the foraging grounds (mixed stock analysis — MSA) we used a Bayesian algorithm with a Markov Chain Monte Carlo (MCMC) method and a hierarchical model (many-to-many; Bolker et al., 2007). We performed four analyses with different assumptions: (*i*) rookery size as prior, (*ii*) rookery size prior and hybrid haplotypes in Brazil, (*iii*) no rookery size prior and no hybrid haplotypes, and (*iv*) no rookery size prior and hybrid haplotypes. For each of the four analyses we considered "*foraging-ground centered*" (FG-centric; the proportion of each rookery contributing to a foraging ground) and "*rookery centered*" (RO-centric; the proportion of each of the rookeries contributing to a feeding area). While the FG-centric can be considered as a mixture proportion in each feeding ground, the RO-centric is a dispersal measure of each rookery. Convergence of MCMC estimates to a desired posterior probability was assessed using the Gelman–Rubin shrink factor (Gelman and Rubin, 1992), increasing the MCMC steps until all values obtained were less than 1.2. The estimated sizes of each population (represented by the number of females/year) were taken from Mortimer and Donnelly (2008). All analyses were conducted in R (R Development Core Team, 2011), using the package *mixstock* (Bolker, 2008). Orphaned haplotypes were excluded from the analyses.

Because of difficulties to use the software STRUCTURE for analyses with linked uniparental markers, such as mtDNA sequences, we used a multivariate method to make assumptions regarding data structure. Mitochondrial haplotypes (384 bp) were imported into R using the package *ape* (Paradis et al., 2004), and only the polymorphic sites were extracted from the sequences. Then, a Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) was performed with the package *adegenet* (Jombart, 2008) to identify and describe sequence clusters. The DAPC relies on data transformation using Principal Component Analysis (PCA) as a prior step to Discriminant

Table 1
Absolute frequencies of control region mtDNA haplotypes described for Brazilian samples from nesting and feeding grounds with the corresponding 384 bp and 740 bp sequence matches from sequences deposited in GenBank and the designation of the haplotype in the literature.

Haplotype (740 bp)	Observed frequencies						Matching short sequences (384 bp)	Matching long sequences (740 bp)
	Feeding	Nesting				Total	ID	
	BR-FEED	BR-BA	BR-RN	BR-SE	BR-CE			
EiBR2		2		2		2	*L. olivacea*[1]	
EiBR3		21			1	23	*C. caretta* EiBR3[1]	
EiBR4		29		1		30	*C. caretta* EiBR4[1]	
EiBR5	3					3		EiA-49[7]
EiBR6	1					1	EATL[3]	
EiBR7	6					6	EATL[3]	
EiBR8	65	53	21	1	1	140	A[4]	A01[9]
EiBR9	2	4				6	A[4]	
EiBR10	6	9				15	f[6], EiBR19[2]	
EiBR12	2					2	b[6]	
EiBR13	2					2	F[4]	A11[9]
EiBR14	1					1	H2–H5[5,8]	
EiBR15	1					1	F[4]	A45[9]
EiBR16	2	1	6			9		
EiBR17	1					1	α[6]	A02[9]
EiBR18	1					1		
EiBR19	1					1	f[6],EiBR10[2]	
Total	94	119	27	4	2	246		

Data sources are from:
[1] Lara-Ruiz et al. (2006).
[2] This study.
[3] Bowen et al. (2007).
[4] Bass et al. (1996).
[5] Al-Mohanna and George (GenBank).
[6] Diaz-Fernandez et al. (1999).
[7] Monzón-Argüello et al. (2010).
[8] Tabib et al. (2011).
[9] Leroux et al. (2012).

Analysis (DA), which maximizes the separation between groups. The optimal number of clusters was predicted using the sequential K-means clustering method, using the Bayesian Information Criterion (BIC) for choosing the best number of groups (K) from 1 to 10. The number of principal components that explained 90% of the cumulative variance was retained.

We used autosomal data available in Vilaça et al. (2012) for the same samples of this study. Four nuclear markers were used: Oocyte maturation factor (Cmos), two recombination activating genes (RAG1 and RAG2), and one intron of the RNA fingerprint protein 35 gene (R35). Data from the BDNF gene were excluded since both hawksbills and loggerheads (*C. caretta*) show the same allele at this locus. Based on the mtDNA results of population differentiation, we also used nuclear sequences to estimate the differentiation among populations and to characterize the past population dynamics using the Extended Bayesian Skyline Plot (EBSP; Heled and Drummond, 2008) implemented in the software package BEAST v.1.6.2 (Drummond and Rambaut, 2007). Even though the impact of population structure in skyline plot methods has not been investigated in great depth, it is likely that it may lead to biased estimates of model parameters (Ho and Shapiro, 2011). The bias in population structure can cause that the demographic plots can reflect changes in the degree of structure rather than changes in the overall population size. Since the data from Vilaça et al. (2012) for *E. imbricata* (n = 121) comprise both foraging (AR and FN) and rookeries (BA and RN), we separated the data in three datasets: the two rookeries (BR-BA and BR-RN) and one foraging area (BR-AR + BR-FN, hereafter referred as BR-FEED). The EBSPs were performed only for the two rookeries, since feeding areas are temporal aggregates of diversely structured populations. Analyses were run for 50,000,000 generations, logging every 1000. Convergence was checked in Tracer v.1.5 to assure all parameters had an ESS (estimated sample size) of at least 200.

3. Results

The examination of nesting (n = 152) and foraging (n = 94) samples increased from 7 to 17 the number of mtDNA haplotypes described for Brazil. All new haplotypes were found exclusively among samples from foraging grounds. Excluding the hybrid mtDNA haplotypes (EiBR2, 3 and 4) derived from *C. caretta* and olive ridley (*Lepidochelys olivacea*) progenitors (Lara-Ruiz et al., 2006), the 14 *E. imbricata* haplotypes are 740 bp long, with 47 variable sites (12 singletons) represented by 41 transitions (Ti) and 6 transversions (Tv). Haplotypes EiBR8, 9, 10, 12, 13, 14, 15, 16, 17 and 19 present a deletion at site number 10, which is not present in haplotypes EiBR5, 6, 7 and 18. Compared to the available *E. imbricata* short haplotypes (384 bp), EiBR6 and 7 matched the EATL haplotype described by Bowen et al. (2007) for one sample from feeding grounds at the US Virgin Islands, but it was recently also found on the nesting site of Principe Island (Monzón-Argüello et al., 2011), and feeding aggregations of Cape Verde and Principe (Monzón-Argüello et al., 2010; Monzón-Argüello et al., 2011). This haplotype is more similar to the Indo-Pacific (Australian) haplotypes than to Caribbean ones. Haplotypes EiBR8 and 9 correspond to haplotype A (Bass et al., 1996), which is one of the two common haplotypes found in the Caribbean, while EiBR13 and 15 correspond to haplotype F (Bass et al., 1996), which is the other most common sequence found in the Caribbean, and also found at the Cayman Islands (Blumenthal et al., 2009). Haplotypes EiBR10 and 12 match haplotypes f and b, respectively, which were previously reported in feeding areas at Puerto Rico (Mona Island) and Cuba (Diaz-Fernandez et al., 1999). Haplotype EiBR19 also matched haplotype f. When considering only the 384 bp available for all sequences in GenBank, haplotype EiBR14 is identical to the corresponding alignment sites of haplotypes H2 and H5 (GI:115371805 and GI:122720664) described in Kuwait (Al-Mohanna and George, unpublished). Haplotype EiBR17 corresponds to a haplotype designated Alpha (α), which was only found in a nesting

colony from Costa Rica (Troeng et al., 2005), and in several feeding grounds in the Caribbean (Bowen et al., 2007; Bowen et al., 1996; Diaz-Fernandez et al., 1999) and Cayman Islands (Monzón-Argüello et al., 2010). Because of the use of shorter sequences, haplotypes EiBR3 and 4, EIBR6 and 7, EiBR8 and 9, and EiBR10 and 19 were binned together in later analyses (see below).

Although few longer sequences of 790 bp are available, the most frequent haplotype in Brazilian rookeries (EiBR8) matches the most common Caribbean haplotype, A01 (Leroux et al., 2012; Velez-Zuazo et al., 2008). Other haplotypes found only in feeding grounds (EiBR13, EiBR15, EiBR17) also matched Caribbean haplotypes (A11, A45, A02, respectively). The haplotype EiBR5 matched the A49 haplotype from Monzón-Argüello et al. (2010) that was described at a feeding aggregation from Cape Verde. The haplotype EiBR14 is identical to a sequence described by Tabib et al. (2011) for Iranian aggregations. Haplotype frequencies for the entire Brazilian sample, together with their similarities to previously described sequences (when considering the 384 bp and 790 bp haplotypes in GenBank) are shown in Table 1.

Including the hybrids (EiBR2, 3 and 4) and the 14 *E. imbricata* haplotypes, the overall mean distance was 0.053. Distance between *C. caretta* and *L. olivacea* haplotypes was 0.09, between *C. caretta* and *E. imbricata* haplotypes was 0.108 and between *E. imbricata* and *L. olivacea* haplotypes was 0.143. Hybrid haplotype EiBR2 is equivalent to haplotype F (Bolten et al., 1998), the most common *L. olivacea* haplotype found in Brazilian rookeries, and haplotypes EiBR3 and EiBR4 are equivalent to CC-A4, the most common and exclusive *C. caretta* haplotype found in the Brazilian rookery (Reis et al., 2010).

The network revealed the presence of four distinct haplogroups (A, F, I-P1, I-P2) in the feeding grounds, while only haplotypes belonging to haplogroup A are present in the nesting grounds (Fig. 2). Sequences in each haplogroup differed among each other for one or two substitutions (average distance within groups, d =0.001–0.003) while a minimum of 10 substitutions separated each of the defined haplogroups (average distance between groups, d =0.017–0.042). Among the described haplotypes, five (EiBR5, 6, 7, 14 and 18) corresponded to sequences more likely related to eastern Atlantic and/or Indo-Pacific samples, representing almost 13% of the feeding ground samples. Only three samples from feeding grounds showed haplotypes related to the F haplotype (EiBR13 and 15), reported to be one of the two most common haplotypes in the Caribbean, while the rest of the samples (including all samples from nesting sites) belonged to the group of sequences related to the other most common Caribbean haplotype (A, described by Bass et al. (1996)).

Based on the DAPC results, the best estimated number of clusters was 5, although the BIC values for four clusters were not much higher (Supplementary Material, Fig. 1). All samples were assigned to its respective cluster with 100% confidence. Group separation was concordant with the divergence already observed in the network (Fig. 3). When K = 5, two highly distinct Pacific groups were found, two Atlantic ones (one represents haplogroup F and another haplogroup A), and another one is composed by a single sequence (BI1 — GenBank number: DQ479341), which is found in the US Virgin Islands and Mexico rookeries. Although BI1 is more related to Atlantic sequences it appeared to be basal in relation to other Atlantic haplotypes in a phylogenetic tree (data not shown). When using K = 4, this haplotype grouped with the haplogroup F. The Pacific group I-P1 clustered with the EATL haplogroup, while the I-P2 grouped the Brazilian haplotype EiBR14 with Iranian sequences, suggesting two different clades in the Indo-Pacific region.

The AMOVA detected significant differentiation among nesting samples from different Brazilian localities (BA × RN), but no significant difference was detected between the two feeding grounds in Brazil (Supplementary Material, Tables 1 and 2). For this reason, all samples from feeding grounds (Fernando de Noronha and Rocas Atoll) were considered to represent a single aggregation (BR-FEED), while the nesting sites from the Brazilian coastline were considered two separate demographic units (BR-BA and BR-RN). Sequences

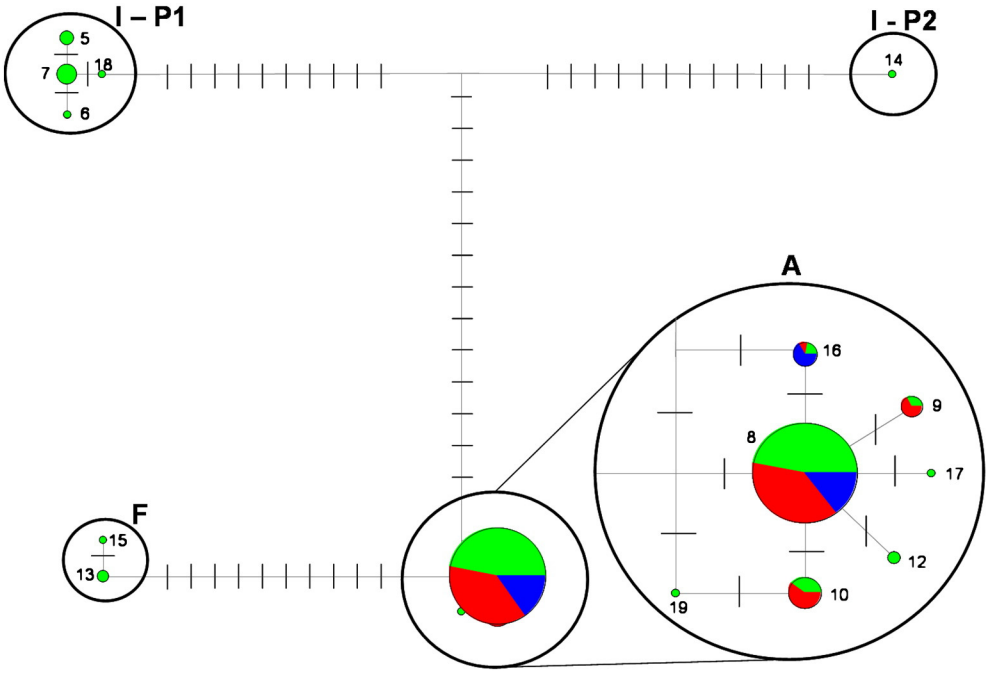

Fig. 2. Median joining network showing the relationships between *E. imbricata* mtDNA control region haplotypes described for Brazilian samples. Haplogroups were named following the most common haplotypes registered in the literature (A and F) or I-P1 for the group related with Kuwait sequences and I-P2 for the sequence that matches the EATL haplotype that has been related with Australian samples. The area of the circle is proportional to haplotype frequencies in all data set. The detail depicts the relationships among haplotypes from haplogroup A. Number of mutations are indicated by transversal bars. Green: samples from feeding grounds (BR-FEED). Red: samples from BR-BA. Blue: samples from BR-RN. For clarity, only the numbers that identify each haplotype were included.

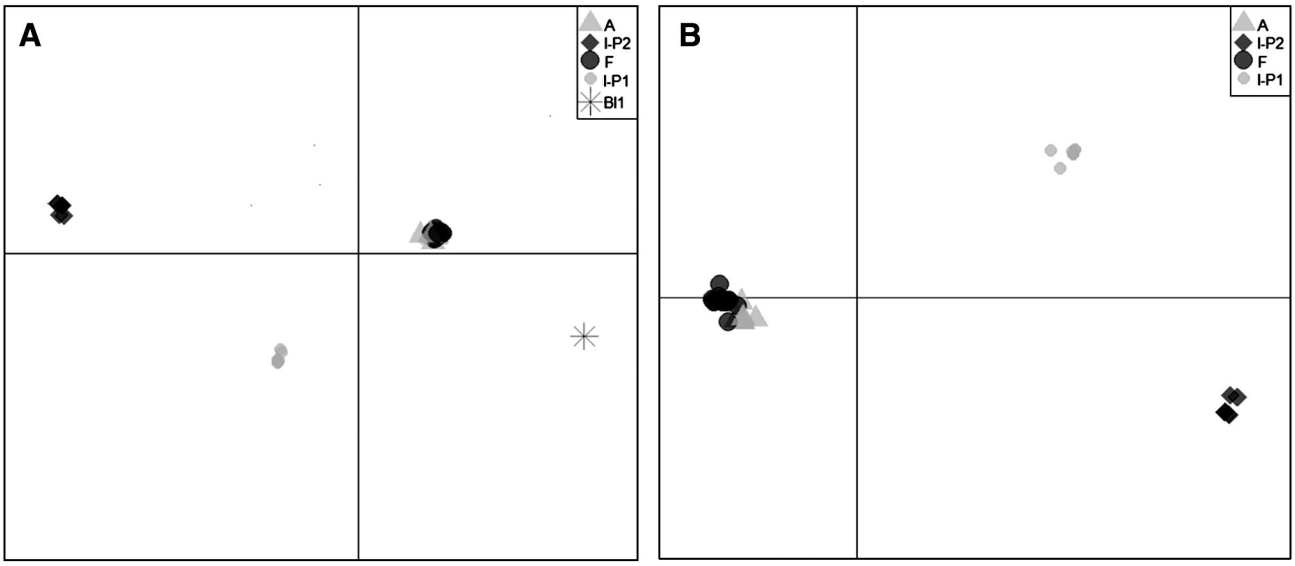

Fig. 3. Discriminant Analysis of Principal Components (DAPC). (a) Scatter plot of DAPC performed with shorter sequences (384 bp) for K = 5. (b) Same analysis with K = 4.

from SE and CE rookeries were not considered due to small sample size. Comparisons between these three units indicated that there are significant differences among them ($\Phi_{ST} = 0.31$ $p < 0.0001$), even when "hybrid" haplotypes from nesting beaches were not considered ($\Phi_{ST} = 0.082$ $p = 0.002$). Diversity indexes (Table 2) were higher for the nesting sample when all haplotypes were considered, but when comparing these indexes without taking into account the hybrids with haplotypes EiBR2 (*L. olivacea*) and EiBR3 and EiBR4 (*C. caretta*), all diversity estimates obtained for the feeding aggregate were higher.

For the nuclear data, we included 59 samples from rookeries (41 for BA and 18 for RN) and 42 samples from feeding aggregations. Rag1 showed three haplotypes, Cmos five haplotypes (one exclusive of feeding areas — Hap6), and R35 with seven haplotypes (one found only in rookeries and two only in feeding areas). Both rookeries had a similar haplotype composition, except for the Cmos gene, the BA population showed six haplotypes and the RN population only one (Hap8). The haplotype frequency for each site is shown in Supplementary Material, Table 3. In contrast to mtDNA results, the nuclear data did not show significant differentiation between the two Brazilian rookeries and the feeding area (Cmos $\Phi_{ST} = 0$, $p = 0.38$; Rag1 $\Phi_{ST} = 0$, $p = 0.54$; R35 $\Phi_{ST} = 0.008$, $p = 0.10$), and generally no differentiation between nesting populations (Cmos $\Phi_{ST} = 0.04$, $p < 0.05$; Rag1 $\Phi_{ST} = 0$, $p = 0.93$; R35 $\Phi_{ST} = 0$, $p = 0.74$). Only Cmos showed a significant differentiation between the two nesting areas BR-BA and BR-RN. The EBSP failed to show any changes in population size (Supplementary Material, Fig. 2). The neutrality tests (Fu's Fs and Tajima's D) were not significant for any population or gene, except for the R35 intron. It showed a significant signal of expansion for the feeding area ($Fs = -1.88$, $p = 0.006$; $D = -2.95$, $p = 0.04$) and Bahia nesting population ($Fs = -1.94$, $p = 0.001$; $D = -6.38$, $p < 0.001$). The gene RAG2 was not considered due to lack of polymorphism (only one allele, Hap5, was found in both rookeries and feeding areas).

When Brazilian populations were included in the data set compiled by Bowen et al. (2007), the AMOVA indicated that there were significant differences among nesting sites ($\Phi_{ST} = 0.54$; $p < 0.0001$), with pairwise Φ_{ST} values for the comparison between the rookery in Brazil and all other Caribbean rookeries ranging from 0.05 (RN vs. Cuba) to 0.99 (RN vs. Principe Island) (all p-values < 0.0001). These differences increased when samples with *C. caretta* mtDNA haplotypes were excluded from the Brazilian sample ($\Phi_{ST} = 0.60$; $p < 0.0001$). The exact test of population differentiation (pairwise comparisons) indicated that Brazilian nesting populations are different ($p < 0.0001$) from all other nesting populations studied. The pairwise comparison of the Brazilian feeding ground with other feeding areas worldwide indicated that the former is significantly different from all aggregations, with Φ_{ST} values ranging from 0.20 (vs Cayman Island) to 0.80 (vs. Principe Island).

The AMOVA results indicated a large and significant divergence between feeding aggregations ($\Phi_{ST} = 0.57$; $p < 0.0001$) and this value increased ($\Phi_{ST} = 0.73$; $p < 0.0001$) when populations were grouped according to geography (Caribbean, Brazil, Cape Verde, Principe Island). The mixed stock analysis (MSA) was run considering the Brazilian rookeries from BA and RN as different sources. The MSAs varied considerably when considering population size as prior and the presence of hybrid haplotypes in BR-BA (Fig. 4). RO-centric MSAs show that Brazilian feeding grounds are mostly composed by BR-BA hawksbills (Fig. 4B, E, H, K). When considering hybrid haplotypes, also a large contribution to unknown feeding grounds is observed (Fig. 4E, K). The rookery BR-RN did not show a major contribution to any feeding ground, and exhibited generally wide confidence intervals (CI) for all estimates (Fig. 4A, D, G, J). The FO-centric MSAs showed that BR-FEED hawksbills disperse within the Brazilian shelf, especially to the BR-BA population (Fig. 4C, F, I,

Table 2
Standard and molecular diversity indexes for nesting (with and without "hybrid" haplotypes considered) and feeding aggregates sampled in Brazil. h = gene diversity, π = nucleotide diversity, k = mean number of pairwise differences.

	Nesting			Feeding
	BR-BA		BR-RN	BR-FEED
	With hybrids	Without hybrids		
N	119	**67**	27	94
No. of polymorphic sites (S)	143	3	1	48
No. of haplotypes	7	4	2	14
Ts	98	3	1	41
Tv	35	0	0	6
No. of sites with indels	15	0	0	1
h	0.712	0.358	0.359	0.516
π	0.0599	0.0005	0.0005	0.0093
k	44.967	0.379	0.359	6.929

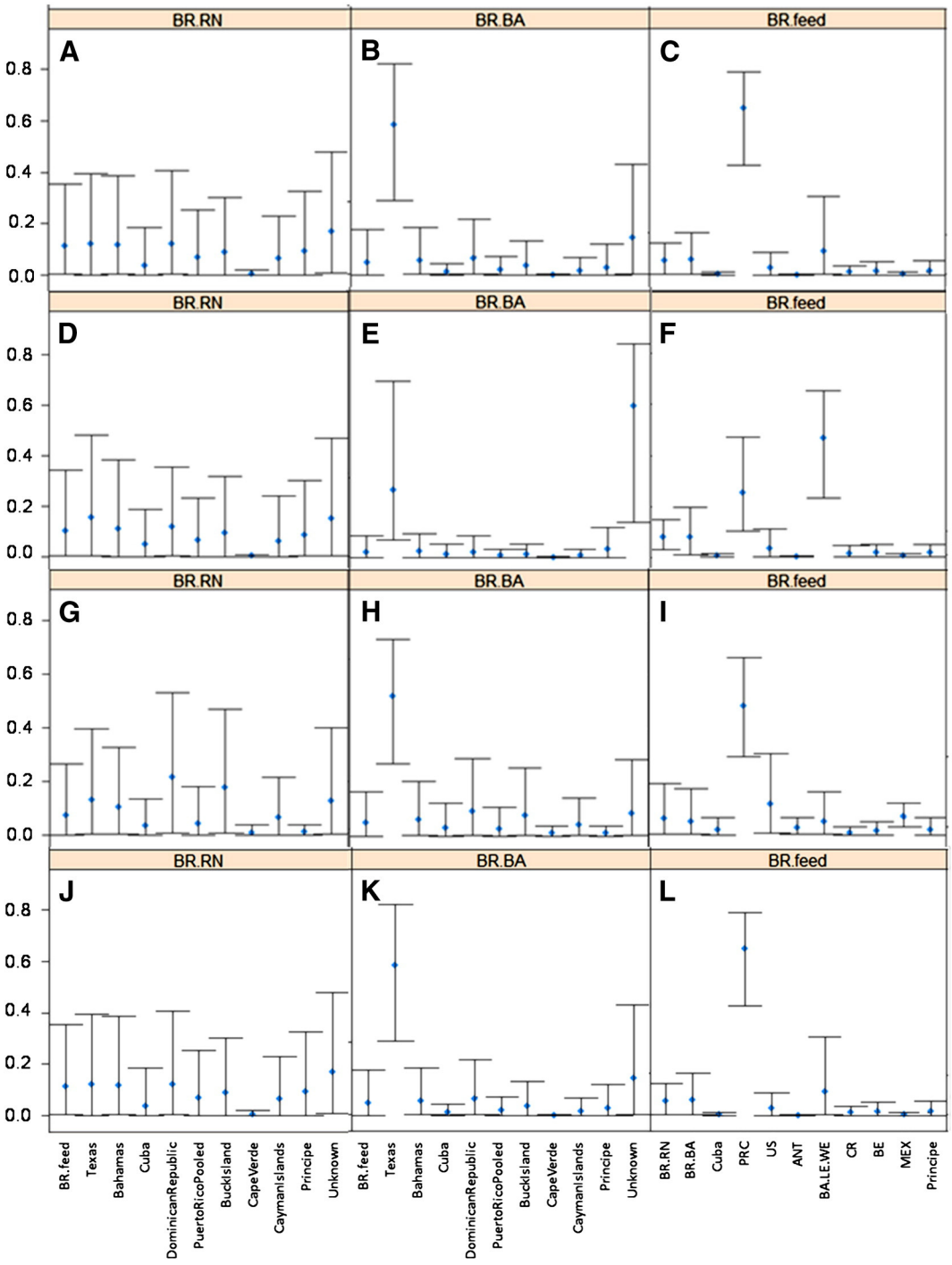

Fig. 4. Mixed stock analysis (many-to-many) results showing mean proportional contribution (dots) and 95% confidence intervals (CI, bars). Results from rookery-centric for BA-RN (first column, A, D, G, J) and BR-BA (second column, B, E, H, K) are shown in columns. Foraging-ground-centric results are shown in graphs (C, F, I, L, third column). Four combination of priors were considered: rookery population size and absence of hybrid haplotypes (A–C), rookery population size and presence of hybrid haplotypes (D–F), no population size and absence of hybrid haplotypes (G–I), and no population size and presence of hybrid haplotypes (J–L). Abbreviations of rookery names: ANT: Antigua, BA.LE.WE: Barbados, BE: Belize, CR: Costa Rica, MEX: Mexico, PRC: Puerto Rico, US: US Virgin Islands.

L). When considering hybrid haplotypes and population size as prior (Fig. 4F), a major contribution from Cuba is also observed.

4. Discussion

4.1. Nesting populations from the Southwestern Atlantic area

Our results showed that the rookeries and the feeding aggregates found in Brazil are distinct demographic units. The nesting populations present few diverging *E. imbricata* haplotypes, all derived (one mutation step) from the most common haplotype (EiBR8), which could be likely the ancestral haplotype for the Brazilian rookery population. The star shaped network obtained with mtDNA haplotypes also suggests a population that has experienced a bottleneck followed by population expansion, likely due to a colonization event. However, Tajima's D and Fu's tests of recent population expansion were non-significant, and the EBSP also failed to show any population size variation. If EiBR8 is the ancestral haplotype for the Brazilian rookery, and considering the greater diversity

of EiBR8-derived haplotypes in the Caribbean, it could be suggested either that the Brazilian population has a Caribbean origin, or that EiBR8 haplotype is the ancestral haplotype for the entire Atlantic (plus Caribbean) population.

The nuclear loci showed no structure and no signal of demographic change (expansion/bottleneck), except for the R35 gene which exhibited a sign of population expansion in the neutrality tests. The lack of structure shown by nuclear loci when compared to mtDNA has already been reported (Bowen and Karl, 2007), however, this is the first study that compares these patterns in hawksbill turtles. Like the females, sea turtle males appear to be also philopatric (FitzSimmons et al., 1997; Wright et al., 2012), including hawksbill males (Velez-Zuazo et al., 2008). Because of that, male gene flow may be not a possible explanation for the lack of structure in nuclear loci. However, turtles appear to show a deceleration of evolutionary rates, diagnosed as a slowdown in mitochondrial mutation rates (Avise et al., 1992) and karyotype evolution (Bickham, 1981; Matsuda et al., 2005). A high level of chromosomal synteny is also observed between turtles, which might be one of the reasons why species separated for millions of years can hybridize (Vilaça et al., 2012). We hypothesize that because sequence changes in nuclear loci have an even slower mutation rate when compared to mitochondria or microsatellites, only a few markers are not informative enough, and only genome-wide studies may recover enough information to identify population structure and demographic changes.

From the four rookery haplotypes (EiBR8, 9, 10 and 16), EiBR10 and 16 differ from haplotype A when shorter (384 bp) sequences are considered, and thus, could be used as population-specific markers. The high frequency of haplotype A, found in all feeding aggregations along the Caribbean, which matches the most frequent haplotype (EiBR8) found in Brazilian rookeries, suggests that at least some of the animals in the Caribbean feeding grounds can have a Brazilian origin. When using longer sequences, the haplotype EiBR8 still matches one of the most common Caribbean haplotypes (A01), which reinforces the connection between Caribbean and Brazilian populations.

The presence of common haplotypes between Brazilian and Caribbean populations suggests a connection between these two areas, but the RO-centric MSA results show that most of Brazilian hawksbills tend to disperse within Brazilian feeding areas. Their contribution to Caribbean feeding grounds varies according to the priors (generally not contributing when population size and presence of hybrids are considered; and contributing when population size is not taken into account). When hybrid haplotypes are considered in the BR-BA, the contribution to "unknown" populations increases, reflecting the fact that we still do not know where hybrids migrate to, since they were not found in high frequencies in any feeding ground (Vilaça et al., 2012). While the confidence interval (CI) range for the BR-BA population varied according to the priors, the BR-RN showed always wider values of CI. BA-BR shares several haplotypes with BR-FEED (including haplotypes that are only found in these two areas), but because BR-RN profile is shared with both Brazilian and Caribbean populations no specific contribution can be delimited for this population.

4.2. Feeding aggregations

As opposed to the rookery populations, the feeding aggregations presented a high number of distinct haplotypes (14 haplotypes, 4 haplogroups) and accordingly, are characterized by high values of nucleotide and haplotype diversity, similar to values reported for other aggregates already studied (Bass et al., 1996; Bowen et al., 2007; Diaz-Fernandez et al., 1999; Troeng et al., 2005). The Brazilian aggregates (FN and AR) present one of the highest numbers of haplotypes registered for a single feeding assembly, only comparable with values found in some feeding aggregates in Cuba (14 haplotypes) and Mona Island (19 haplotypes) (Diaz-Fernandez et al., 1999; Velez-Zuazo et al., 2008).

Bass et al. (2006) suggested that feeding areas located within the confluence of major current systems exhibited higher haplotype diversity compared to other aggregations and the feeding aggregations of FN and AR are located in the confluence of two great currents: the North Brazilian Current and the South Equatorial Current. Other studies have raised the possibility of great influence of currents in the routes of marine turtles (Bass et al., 2006; Blumenthal et al., 2009; Gaspar et al., 2012; Marcovaldi et al., 2011), including trans-Atlantic migrations that could be facilitated by the South Equatorial Current, which passes by the two Brazilian feeding aggregations.

The finding of haplotypes EiBR13 and 15 (Table 1), related to the F (Puerto Rico, Cuba, Virgin Islands and Belize) and the EiBR17 related to Alpha (Costa Rica) haplotypes described for Caribbean nesting colonies (Bass et al., 1996; Diaz-Fernandez et al., 1999) provides a possible link between the Caribbean rookeries and Brazilian feeding areas, highlighting the importance of Brazilian feeding aggregates for the conservation of Caribbean populations. Even when considering longer sequences, the link between these two areas are confirmed, with haplotypes from Caribbean rookeries being found in Brazilian feeding grounds. It also reinforces the hypothesis that these animals are capable of migrating long distances between their natal areas and developmental habitats (3500 km in a straight line from Barbados – 6000 km from Costa Rica following the coastline). The link between Brazilian feeding areas and Caribbean populations is reassured by the MSA results that indicated around 30% (20% when hybrids are considered and somewhat more when hybrids are excluded) of the feeding aggregates to be composed by individuals from insular Caribbean rookeries. The contribution of the coastal Caribbean rookeries is lower, which is expected because of the oceanic currents, and the higher contribution of the Brazilian rookery to feeding grounds in FN and AR is also expected due to the short distances between rookery and feeding areas in Brazil (ca. 540 km).

Interestingly, almost 15% of the samples from the Brazilian feeding aggregates have a putative Eastern Atlantic or Indo-Pacific origin, reinforcing the idea that *E. imbricata* juvenile trans-Atlantic migrations could be more common than was previously thought. Recaptures of hawksbills tagged by TAMAR in Fernando de Noronha have been reported in Gabon (Africa), a straight line distance of ~4200 km (Bellini et al., 2000); and also Corisco Bay (Africa), a straight line distance of ~4796 km (Grossman et al., 2007). One hawksbill tagged in Rocas Atoll was also recaptured in Senegal (Africa), ~2700 km apart, and only 176 days after tagging (Projeto-TAMAR, unpublished results). If the animals are coming from African rookeries, they must be traveling at least 2500 km, but if they come from the Indo-Pacific as suggested by our DAPC, the distance could rise to more than 10,000 km.

Studying the migration pattern of *Chelonia mydas* juveniles, Hays et al. (2010) suggested that the selection of a permanent foraging site is influenced by drifting during the pelagic phase, when movements of hatchlings are greatly influenced by oceanic currents. Based on the sites visited during the drifting period, they imprint their permanent foraging site, the one to be visited in later life stages. Considering Hays et al. (2010) results, the South Equatorial Current may be determinant on trans-Atlantic migrations, and turtles coming to feed on the Brazilian aggregations arrive due to the influence of oceanic currents during the pelagic phase. Although eastern Atlantic rookeries investigated so far possess EATL-like haplotypes (Monzón-Argüello et al., 2010; Monzón-Argüello et al., 2011), which can explain the presence of this haplogroup in the Brazilian feeding aggregations from individuals migrating from Eastern Atlantic rookeries, one haplotype found in Brazil was identical to an Iranian haplotype (both 340 bp and 740 bp), suggesting trans-oceanic migrations. In these migrations, the turtles may take the currents that connect the Indian and Pacific oceans to the Atlantic Ocean, circumventing the African continent. *E. imbricata* trans-oceanic migrations were previously

suggested in the Principe Island (Monzón-Argüello et al., 2011). Because nesting aggregations in eastern Atlantic exhibit EATL haplotypes, the presence of this "typical" Indo-Pacific haplogroup in Atlantic waters does not necessarily mean that *E. imbricata* individuals are currently migrating among oceans. Instead, it is likely that we detected a recent migration, since a new Indo-Pacific haplogroup was found (I-P2), and the only sequence known in literature that matches this haplotype found in Brazilian feeding aggregations is an Iranian one.

As registered elsewhere (Bass and Witzell, 2000; Bass et al., 2006; Bowen et al., 2007; Luke et al., 2004), large nesting colonies are expected to contribute more than smaller ones, and closer ones more than distant ones, but the relative effect of distance is not so clear (Bowen et al., 2007), with oceanic currents playing a major role on the distribution of genetic diversity (Blumenthal et al., 2009). The closest rookeries in Brazil (BR-BA and BR-RN) contribute in a greater extent to the Brazilian feeding aggregation, indicating that in this particular case, smaller distance is more important than rookery size. However, in a micro-regional scale, BR-BA is a much larger nesting colony than BR-RN, and is also suggested to contribute with more juveniles to the Brazilian feeding aggregate sites (FN and AR), although the absence of hybrids indicate the contrary.

Even though the MSA results have to be considered with caution because of wide confidence interval values, they are comparable and often lower than other values registered in the literature. The wide CI ranges obtained in the Bayesian procedures to estimate stock composition are associated to the sharing of haplotypes between rookeries, which reflects less confident conclusions. In this case, haplotype A is the most common for rookeries in Brazil, Barbados, Cuba, and Antigua. However, other factors should explain the low contribution from Brazilian rookeries to FN and AR feeding grounds found by the MSA with hybrids when compared to without the hybrid haplotypes. The hybrid haplotypes (EiBR3 and 4) account for almost 40% of the rookery samples, while EiBR8 represents 50% of these samples. Thus, if the contribution to the feeding grounds was mainly from the Brazilian rookery in Bahia (BA), it would be reasonable to expect at least a similar proportion of hybrid and EiBR8 haplotypes in the feeding aggregate. Thus, Bayesian estimates of the Brazilian rookery contribution were highly influenced by the absence of hybrid haplotypes in the feeding ground. This estimate could have been lower if it was not for the presence of two haplotypes exclusively found in the Brazilian nesting population, which were also found in the feeding grounds (EiBR10 and 16).

However, when the MSA analysis was run disregarding the hybrid haplotypes from Brazilian rookeries, the estimate for their contribution to the feeding ground rises up to 69% (when using population size prior, and 53% without size prior), with narrower CI ranges. This high contribution is expected since all non-hybrid haplotypes present in the Bahia rookery also appear at the Brazilian feeding aggregates, a situation not observed for any other rookery used in the analyses. These contrasting results demonstrate the sensitivity of the MSA analysis to the presence or absence of haplotypes in the prior (baseline) data from the rookeries. In the case of the analysis including hybrid haplotypes, the absence in the feeding sites of one of the high-frequency haplotypes from the Brazilian rookery leads to a low contribution estimate. In light of our findings, this result could suggest that: *i*) most of the hybrid individuals born in Brazil do not migrate to the feeding areas sampled during this study, or *ii*) only the non-hybrid animals from the Brazilian rookery reach these feeding areas. If the latter situation is the case, then there are two likely explanations for this. First, it is possible that only the closest nesting population (RN), which presents no hybrids, is contributing young individuals to Fernando de Noronha (FN) and Rocas Atoll (AR) feeding sites, although the MSA results suggest the contrary. The MSA results show a low contribution of RN to feeding sites, likely due to the smaller number of nesting females per year when compared to BA, and also to the haplotype composition of RN, which has a great contribution of haplotype EiBR16 that is not found in the feeding aggregation. However, since our results show that the distance from the foraging site is an important factor, the individuals breeding in RN (and surrounding nesting sites not analyzed in this study) may contribute to a larger extent to FN and AR feeding sites. Second, the largest nesting population in Brazil where the hybrids occur (BA) also contributes to FN and AR, but hybrid individuals display different feeding or migratory behavior, keeping them away from the hawksbill juvenile feeding areas in FN and AR. A recent study (Marcovaldi et al., 2012) showed that, indeed, the adult hybrid females show different migration behavior, migrating either to *C. caretta* or *E. imbricata* adult feeding areas. Six hybrids were satellite-tracked in this study, and only one of them showed a typical migration pattern of *E. imbricata*, while three migrated to *C. caretta* feeding sites. If hybrids are escaping the outbreeding depression that is expected after the interbreeding of species with very different adaptations, and are not found using the parental species' niche, they could be fitting the *C. caretta*'s niche, or may be capable to explore an even wider niche (Marcovaldi et al., 2012) as shown by different migration patterns. The factors that lead to a hybrid to behave as one or another species (or maybe both) are yet to be investigated.

A recent large survey on Brazilian sea turtles (Vilaça et al., 2012) found one *E. imbricata* × *C. caretta* hybrid (>F1) on Rocas Atoll that was previously identified as *E. imbricata*. However, it failed to find any *E. imbricata* × *C. caretta* hybrids on another important *C. caretta* oceanic feeding site (Rio Grande Elevation). Another *E. imbricata* × *L. olivacea* hybrid individual was recently found in the Caribbean (Richardson et al., 2009), composing the only two hybrids found so far in feeding aggregates in the Atlantic. The absence of hybrid haplotypes in other studied feeding aggregates indicates that the contribution of hybrids from Brazilian nesting beaches, at least from the Bahia (BA) rookery, to the studied Brazilian and Caribbean foraging assemblages seems to be unlikely, unless hybrids display a very peculiar behavior on feeding preferences and migration patterns.

The absence of hybrid haplotypes in other Brazilian rookeries (Vilaça et al., 2012) confirms once again the natal homing and spawning site fidelity of these animals, and indicates that the hybridization process is local (Bahia in Brazil). Satellite telemetry data (N = 15) have shown that adult *E. imbricata* females (hybrids and non-hybrids) nesting in Bahia State, move either northwards or southwards after the breeding season but tend to remain in the Brazilian continental shelf (Marcovaldi et al., 2012). These results suggest that adult turtles nesting in the Brazilian coast apparently do not migrate to feeding sites beyond the Brazilian continental shelf, which could indirectly support the idea that most of the young individuals found in Fernando de Noronha and Rocas Atoll feeding aggregations may be also derived from Brazilian nesting sites.

Because MSA analysis is incapable to deal with orphaned haplotypes, they were excluded from the analysis. However, the proportion of these haplotypes is considerable (14.9%), and if they have an Indo-Pacific or Western Atlantic origin, long trans-oceanic migrations might not be as infrequent as it was believed before. The multiple origins of the individuals at these feeding grounds imply that harvest and/or incidental capture of animals in Brazilian feeding grounds could be affecting several nesting populations around the world, besides the Caribbean.

4.3. Implications for conservation

Our results revealed striking differences between the two Brazilian nesting sites studied. The two rookeries are inserted within the Southwest Atlantic Regional Management Unit (RMU) (Wallace et al., 2010), and belong to related but different genetic stocks, which should be the target of constant conservation efforts. These two areas alone contribute with more than 70% of the juveniles observed

within this RMU (TAMAR, unpublished data). However, there is still little evidence of connectivity between the Brazilian rookeries and feeding aggregates elsewhere, although a small contribution to the Caribbean and eastern Atlantic feeding grounds could be inferred. The results also highlight the importance of the main Brazilian feeding aggregates found in Rocas Atoll (AR) and Fernando de Noronha (FN) for worldwide conservation purposes, since these are developmental areas where juveniles are recruited to spend several years until they migrate to other adult feeding areas or to nesting sites. The possibility of long trans-oceanic migrations implied by hawksbills has to be considered due to the likely occurrence of Indo-Pacific or Western Atlantic mtDNA lineages in Brazilian feeding grounds. Thus, harvest in these feeding areas could affect considerably the nesting populations in the Caribbean and Brazil, but also other populations from western and eastern Atlantic and Indo-Pacific regions. Fortunately, both feeding areas are protected biological reserves in Brazil.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2013.06.004.

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