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Extensive hybridization in hawksbill turtles (*Eretmochelys imbricata*) nesting in Brazil revealed by mtDNA analyses

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Abstract

Bahia state hosts over 90% of hawksbill (*Eretmochelys imbricata*) nests registered in the main nesting sites monitored by Projeto Tamar-IBAMA in Brazil. The genetic diversity of this hawksbill population (n = 119) was assayed through the analyses of 752 bp of the mitochondrial DNA control region in nesting females. Seven distinct haplotypes, defined by 125 polymorphic sites, were found. Most of the individuals (n = 67) display four typical hawksbill haplotypes, 50 individuals display two haplotypes characteristic of the loggerhead turtle (*Caretta caretta*) and two individuals had a haplotype affiliated with the olive ridley (*Lepidochelys olivacea*). These results demonstrate hybridization between the hawksbills and two species that nest along the Bahia coast. Of special interest is the high occurrence of loggerhead × hawksbill hybrids (42%), which display loggerhead mtDNA haplotypes but are characterized morphologically as hawksbills. The true hawksbill haplotypes present only three variable sites and low genetic diversity values ($h = 0.358 \pm 0.069$; $\pi = 0.0005 \pm 0.0001$). The occurrence of several nesting individuals with identical mtDNA from another species may also suggest a long history of introgression between species producing likely F2 or further generation hybrids. Marine turtle hybrids have been previously reported, but the high frequency observed in Bahia is unprecedented. Such introgression may influence evolutionary pathways for all three species, or may introduce novel morphotypes that develop apart from the parental species. The presence of a unique hybrid swarm has profound conservation implications and will significantly influence the development and implementation of appropriate management strategies for these species.

Introduction

Sea turtles nesting in Brazil have suffered under prolonged anthropogenic pressure which has caused the decline of all five species that use Brazilian beaches as nesting grounds. Under IUCN criteria, the loggerhead turtle (*Caretta caretta*), the olive ridley (*Lepidochelyes olivacea*), and the green turtle (*Chelonia mydas*), are currently considered "endangered" (EN), while the leatherback turtle (*Dermochelys coriacea*) and the hawksbill turtle (*Eretmochelys imbricata*) are classified as "Critically Endangered" (CR) (IUCN 2004). The hawksbill turtle has a circumglobal distribution in tropical areas of the Atlantic, Indian and Pacific Oceans (Groombridge and Luxmoore 1989; Pritchard and Mortimer 1999). In Brazil, slaughter of nesting females, egg poaching, traffic of shell ornaments, coastal development, and incidental capture by fisheries have reduced the species almost to extinction (Marcovaldi et al. 1999).

Hawksbill nesting in Brazil occurs mostly during the austral summer, generally from December to February, with an average of 800 nests per season. The State of Bahia, where this study was carried out, harbors ca. 90% of all hawksbill nests registered in Brazil. During the same period (1999–2002), three other species nested in northern Bahia, representing 54.8% of loggerhead nests in Brazil (ca. 2600 nests per season in Bahia), 21% of the olive ridley (ca. 600 nests per season in Bahia), as well as some sporadic (ca. 30 nests per season in Bahia) green turtle clutches (Projeto TAMAR data bank).

Molecular markers have proven useful for resolving migration patterns, feeding ground population composition, natal homing, and the genetic composition and structure of rookeries worldwide (Bass et al. 1996; Fitzsimmons et al. 1997a, b; Bolten et al. 1998; Bowen et al. 2005). Hawksbill genetic studies, along with flipper tagging, re-capture and satellite telemetry analyses, have suggested the common use of habitats by different populations throughout the Caribbean and have provided useful information to the understanding of the species biology (Troëng et al. 2005). In Brazil, mtDNA analyses of hawksbills are restricted to the works by Bass et al. (1996) and Bass (1999) examining 14 individuals from two nesting areas in Bahia (Arembepe and Praia do Forte). These preliminary analyses revealed six haplotypes (384 bp), and a high proportion (10 of 14 samples) of loggerhead × hawksbill hybrids (morphologically diagnosed hawksbills with loggerhead mtDNA haplotypes). Evidence of hybridization was first reported by Conceição et al. (1990), who identified a likely loggerhead×hawksbill hybrid in this same population using protein electrophoresis. Despite this hybridization reports, a study on loggerheads from nesting grounds in Brazil did not registered any hawksbill mtDNA haplotypes in 81 loggerhead samples (Soares 2004).

Here, we report the distribution and frequency of interspecific hybrids among hawksbills nesting in Bahia, Brazil, evaluated using mtDNA markers. However, the uniparental nature of mtDNA limits the inferences that can be made about this ongoing hybridization process, highlighting the need to further analyze this population using biparentally inherited nuclear markers.

Methods

During the nesting seasons of 1999/2000, 2000/2001, 2001/2002 and 2004/2005, 117 tissue samples from nesting females and two stranded males were collected by TAMAR field staff in Bahia. The collectors are trained to identify by means of morphological diagnostic characters the different species that can be encountered at nesting beaches (based on international standards described at Eckert et al. (1999)). Samples were collected using a 6 mm disposable biopsy punch, along three nesting beaches: Arembepe (n = 58), Praia do Forte (n = 53) and Costa do Sauípe (n = 8) (Figure 1). For individual identification, each turtle was tagged on the front flippers with Inconel tags (National Band and Tag Co., style 681). Sample processing, sequencing and sequence analyses were carried at the Laboratory of Biodiversity and Molecular Evolution (LBEM) at the Federal University of Minas Gerais (UFMG), Brazil. DNA extraction was performed following the standard phenol/chloroform procedure (Sambrook et al. 1989) with some modifications (detailed protocols available at http://www.icb.ufmg.br/~lbem/protocolos). PCR was performed in an Eppendorff Mastercycler gradient machine, using primers LCM15382 and H950 (F.A. Abreu-Grobois, personal communication[1]) with an amplification profile of 5 min at 94 °C, followed by 36 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. The amplicons (~1000 bp) encompassing a portion of the $tRNA^{Thr}$, the $tRNA^{Pro}$ and a ~800 bp fragment of the control region were purified using Polyethylene Glycol 8000 20% – NaCl 2.5 M. Sequencing was conducted with the ET Dye terminator Cycle Sequencing Kit (Amersham Biosciences) following the manufacturer's recommendations for sequencing in an automated MegaBACE 1000 DNA analysis system. Sequences were read at least twice with both forward and reverse primers. For each sample, the consensus sequence for all reads was generated using the programs Phred 0.020425 (Ewing et al. 1998), Phrap 0.990319 (Green 1994) and Consed 12.0 (Gordon et al. 1998). Consed 12.0 and Sequence Analyzer 3.0 (Amersham Biosciences) were used to

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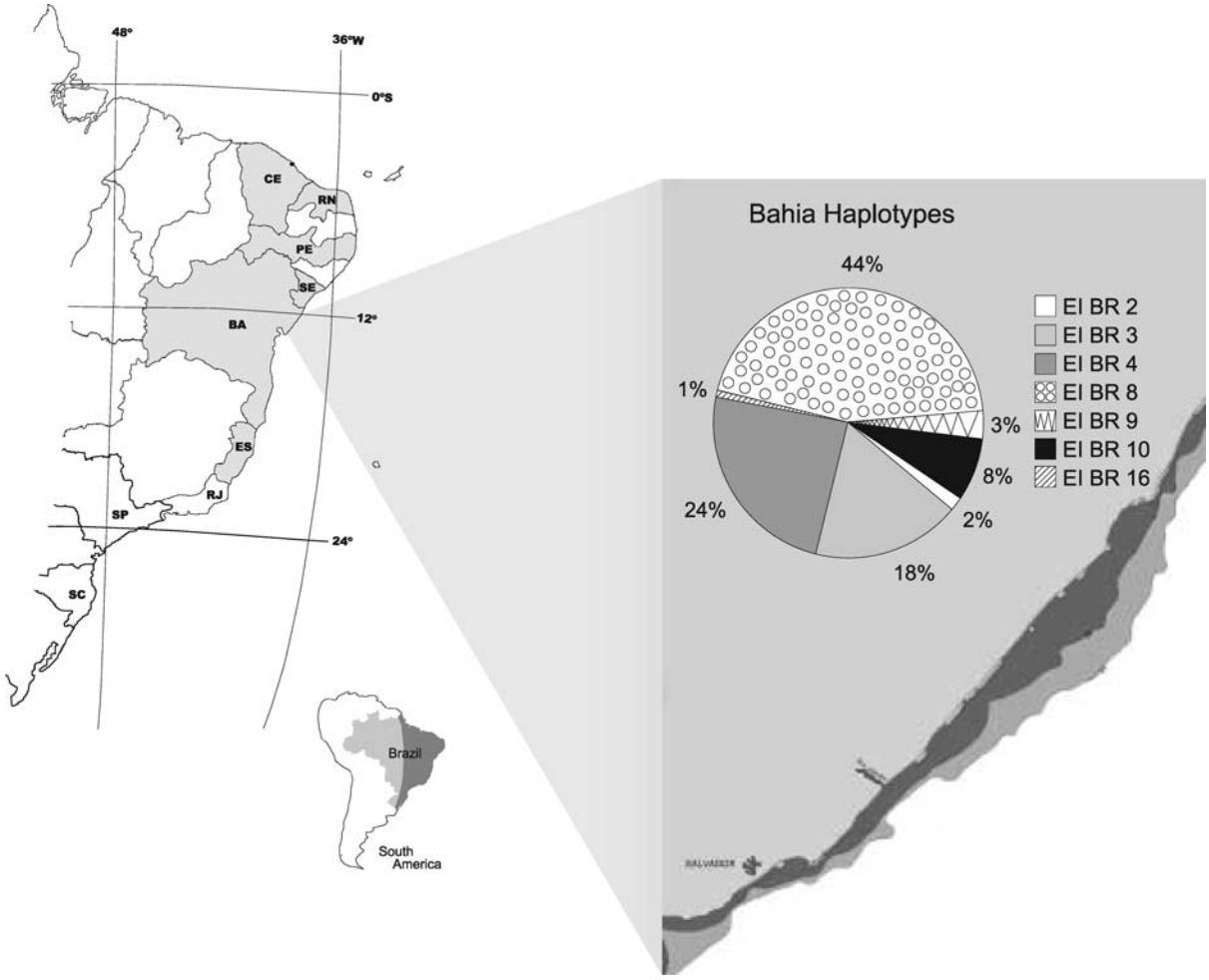

Figure 1. Haplotype frequencies distributed along three nesting sites of *E. imbricata* in the northeastern Brazilian coast.

visualize the chromatograms and verify the quality of the sequences and the base assignment in the observed polymorphic sites. Defined haplotypes start at the first site after the $tRNA^{Pro}$ and encompass about 752 bp of the control region left domain. Sequences were compared with control region sequences of Cheloniidae species (Bass et al. 1996; Diaz-Fernandez et al. 1999; Alberto Grobois, personal communication & Archie Carr Center for Sea Turtle Research: http://www.accstr.ufl.edu/ccmtdna.html). The phylogenetic relationships between haplotypes were determined by Neighbor-Joining and Parsimony methods using MEGA 3.0 (Kumar et al. 2001) with 1000 bootstrap replications, and molecular diversity indexes were calculated using DNAsp 4.0 (Rozas et al. 2003).

Results

The control region sequences obtained in Bahia samples revealed the presence of seven distinct haplotypes (EimBR 2, 3, 4, 8, 9, 10 and 16; Haplotypes EimBR 5–7 and 11–15 were not found in nesting grounds in Bahia and will be presented elsewhere) that are deposited in GenBank under Accession Nos. DQ177335–DQ177341. The polymorphic sites that characterize the seven haplotypes are depicted in Table 1.

The phylogenetic comparison between some of the published sea turtle control region sequences and the seven haplotypes found in this study revealed that only four of them are true hawksbill sequences (Figure 2). For this analysis all hawksbill sequences available in October/2005 in the

Table 1. Polymorphic sites among seven mtDNA control region haplotypes obtained from 119 *E. imbricata* individuals sampled in Bahia (Brazil) nesting grounds

Haplotype	Polymorphic site number
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3
	2 2 2 2 3 3 3 4 5 5 5 6 6 6 7 7 7 7 7 8 9 1 1 2 2 3 3 3 3 4 4 4 5 5 6 6 6 8 8 9 9 9 9 0 0 1 1 1 2 3 4 4 4 4 5 8 9 2 3 3
	1 6 8 1 2 3 8 5 6 9 4 1 4 9 5 8 9 1 2 4 5 8 6 8 0 1 2 8 0 1 4 5 3 6 8 0 8 4 8 9 7 9 0 1 3 8 6 9 1 6 8 6 9 5 6 7 8 9 1 9 1 0 1
EimBR8	A G A A C A C A T C A C G C T G C A C C T C C T A C C G A C C T C A C A A T G G A A C T C C C G A G A G T G T A A A G G T G T
EimBR9	. . . . .
EimBR10	. . . . . G . . . . .
EimBR16	. . . . .
EimBR3	. A . . T G T . . T G . A A . . T . T . C T T A . . T A G T T C T G T T . . A A T G T . . . . . A A A C C . C A . C . .
EimBR4	. A . . T G T . . T G . A A . . T . T . C T T A . . T A G T T C T G T T . . A A T G T . . . . . A A A C C . C A . C . .
EimBR2	G A G G T . . T A T G A A A G A T C T T C T T . G T T A . T T C T G T T . C A A T . . C A A A A G T G A A A C C G C T A . A C

Haplotype	Polymorphic site number *(continued)*
	3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 7 7 7 7
	5 6 6 6 6 6 7 7 2 2 2 2 2 2 2 3 4 4 4 4 5 6 9 0 1 1 1 6 8 8 8 9 9 9 0 1 2 2 2 2 3 3 4 5 6 6 7 7 9 0 0 0 0 1 1 1 1 1 2 2 2 3 3
	5 3 5 6 7 9 0 1 1 3 4 6 7 9 8 1 3 4 7 3 0 1 2 0 1 7 5 3 4 8 1 3 8 8 1 0 7 8 9 2 4 9 9 0 2 3 7 8 0 6 7 9 0 1 2 3 4 2 3 6 3 4
EimBR8	G A T T T T G G G A T G G A T A C T T G C A C A G T A G A A G A A T G T A G A A C A G G A C T T C C T A C C T A T T A C G T
EimBR9	. . . . . A . . . . .
EimBR10	. . . . .
EimBR16	. G . . . . .
EimBR3	A . G . C . A T A G A A A . C G T C C A . . T C . . G A G G . G G C A C G A . T T . T T G T . C . A A . . . C . . C . . . .
EimBR4	A . G . C . A T A G A A A . C G T C C A . . T C . . G A G G . G G C A . G A . T T . T T G T . C . A A . . . C . . C . . . .
EimBR2	. . A C . C . A A G G A A G . G T C C A T G . C A C G A . . T G G C A . G A T T T G T T . T A C T A A C T T A T A C G T A C

GeneBank were used, while the sequences from other species were selected based on several characteristics, including size (bp), geographic origin of the samples, and data about common and rare haplotypes. To simplify Figure 2, only one characteristic haplotype was included for most of the species in the family Cheloniidae (excluding *E. imbricata* and *C. caretta*). The *D. coriacea* (Family: Dermochelidae) haplotype was used as the outgroup. Haplotypes EimBR 8, 9, 10 and 16 are analogous (comparing 339 bp) to typical hawksbill haplotypes found by Bass et al. (1996) in Brazil, forming a distinctive lineage (Figure 2). Haplotypes EimBR8 and 9 exactly match the A haplotype in Bass et al. (1996) and differ by one substitution at the position 660 of our alignment (Table 1). Haplotype EimBR10 is different from them (and from A haplotype in Bass et al. (1996)) by one substitution at position 158 while haplotype EimBr16 has a substitution at position 363 of our alignment (Table 1). Neither EimBr10 nor EimBr16 were found by Bass et al. (1996) in any of the studied populations, so this haplotypes might be exclusive of the Brazilian nesters. The group formed by these haplotypes and the ones found by Bass et al. (1996) in Caribbean and Brazil populations, cluster with a group formed by the four haplotypes from the Red Sea (EimRS1 to RS4, Figure 2).

Haplotypes EimBR3 and EimBR4 differ from each other by one substitution at position 620 of our alignment (Table 1) and are closely related to typical loggerhead haplotypes found at Brazilian nesting populations (Bahia and Espirito Santo), being identical to haplotype D (Bolten et al. 1998) (current nomenclature at Archie Carr Data Base is CC-A4). Haplotypes CC-A24 and CC-A25, found only in Brazilian loggerheads (Soares 2004) differ from CC-A4 by one substitution. These, and haplotypes EimR, S, T and U defined by Bass et al. (1996) as the Brazilian hawksbill × loggerhead hybrids all cluster together.

The haplotype named EimBR2 clustered within the olive ridley clade, being identical to haplotype F defined by Bowen et al. (1997) as the only haplotype present in their Brazilian sample ($n = 15$) (Figure 2) and one of the two (E and F) found in Atlantic Populations.

The typical hawksbill haplotypes (EimBR 8, 9, 10 and 16) were found in 56% (67 out of 119) of the sampled individuals (Figure 1). The most common haplotype, EimBR8, was found in 44% of the samples, including the two males sampled in Arembepe. Haplotypes EimBR3 and EimBR4, related to the loggerhead sequences, comprise 42% of the studied individuals (Figure 1) (EimBR3, $n = 21$; EimBR4, $n = 29$), suggesting that we are observing hybrids of second (F2) or further

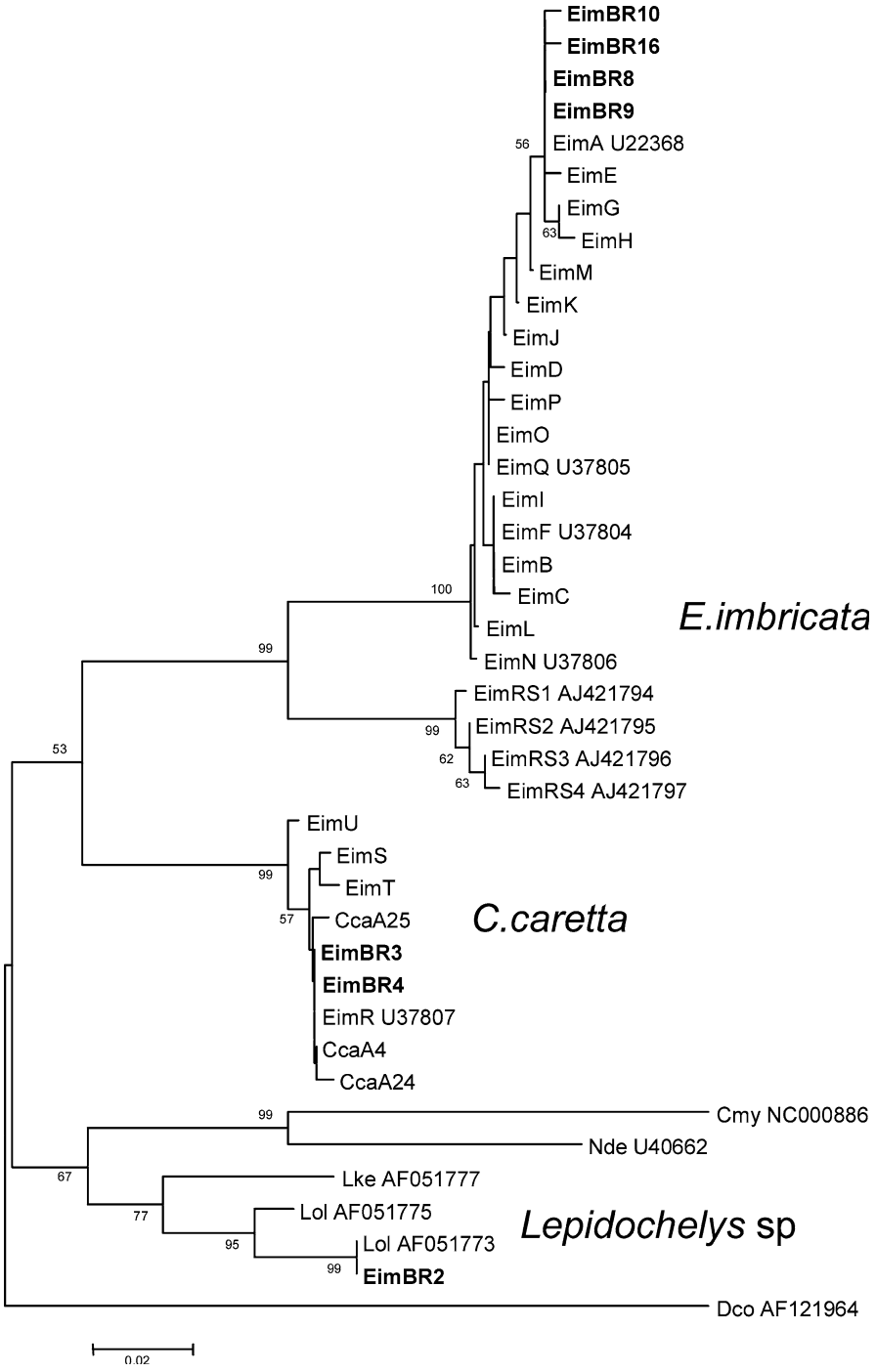

Figure 2. Neighbor-joining tree produced from a 339 bp alignment of D-loop sequences from all sea turtle species. Brazilian haplotypes from *E. imbricata* reported here (EimBR 2, 3, 4, 8, 9, 10 and 16, in bold) are compared to other published haplotypes (Eim A to U) obtained from Bass et al. (1996), or downloaded from the Archie Carr Center for Sea Turtle research database (Cca haplotypes) and from the GenBank (haplotype names followed by accession numbers). Bootstrap support values (>50%) are shown on the branches. Identical tree topologies were obtained using other methods like parsimony (data not shown). Dco (*D. coriacea*), Cmy (*C. mydas*), Nde (*N. depressor*), Lol (*L. olivacea*), Lke (*L. kempii*), Cca (*C. caretta*), Eim (*E. imbricata*).

generations. Only two individuals had the haplotype EimBR2 affiliated with olive ridleys, and both of them were suspected to be hybrids (based on morphological characters) suggesting that the hybridization between olives and hawksbills can be a recent and less widespread event.

These results indicate a high frequency (52 out of 119) of hybrids between hawksbill and other species of the family Cheloniidae occurring in Brazilian nesting grounds. Arembepe was the locality with the highest proportions of hybrids, and the only beach where the low frequency of *E. imbricata* × *L. olivacea* hybrids were detected.

Standard molecular diversity indexes were high, as expected, when calculated for the entire group of samples (Table 2), but decrease when only the hawksbill haplotypes are considered, revealing low levels of genetic diversity in the hawksbill populations that nest along the Bahia coast ($h = 0.358 \pm 0.069$; $\pi = 0.0005 \pm 0.0001$).

Discussion

After 25 years of protection by Projeto Tamar (Brazilian Sea Turtle Conservation and Protection Program), there seems to be an increase in the number of nesting turtles along the Brazilian coast. However, genetic studies on loggerhead (Soares 2004), olive ridley (L. Fernandez, personal communication) and leatherback (P. Dutton, personal communication) nesting populations in this country show low genetic diversity indices. The same seems to be the case for the hawksbill population in Brazil as well, when only true hawksbill haplotypes are considered (Table 2). Diversity indices h (haplotype diversity), π (nucleotide diversity) and k (mean number of pairwise differences) are high when calculated for the entire sample ($h = 0.71$, $\pi = 0.05$), being similar to the highest values found by Bass et al. (1996) among seven sampled Caribbean and one Brazilian population. In Bass et al. (1996) study, only the Puerto Rico population had a higher h value (0.78) than their Brazilian sample (0.70) that included 10 hybrid haplotypes (out of 14 samples). The π value found by these authors for the Puerto Rico population (0.006) is similar to the values found for other populations that did not presented hybrids, and is one order of magnitude smaller than the values found for the Brazilian population in that study (0.025) and in ours (0.05) showing that the hybrid contribution raise this diversity parameter. On the other hand, when only the hawksbill haplotypes are considered, our diversity estimates decrease ($h = 0.36$; $\pi = 0.0005$) being comparable to the low values found by Bass et al. (1996) for Mexico population ($h = 0.23$; $\pi = 0.0003$) and the Virgin Islands population ($h = 0.12$; $\pi = 0.0012$).

Our results indicate high levels of hybridization in the state of Bahia, especially with loggerhead turtles; however, they also report previously unknown hybridization with the olive ridley. Similar hybridization events have already been reported for sea turtle populations (Bowen and Karl 1996) and earlier studies have also suggested this phenomenon for the same area (Conceição et al. 1990; Bass et al. 1996), based on limited sample sizes. Hybridization events are being described for many different fauna and flora taxa, and there is a general concern about the main forces leading to these processes (Rhymer and Simberloff 1996). Anthropogenic factors such as exotic species introduction, and habitat destruction and fragmentation, are primary factors contributing to these phenomena (Rhymer and Simberloff 1996).

Table 2. Standard diversity indexes calculated for each nesting beach (*Arembepe, Praia do Forte* and *Sauípe*) from Bahia (Brazil), for the entire sample (*overall*) and for the sample without the hybrid haplotypes (*Eim**). S, number of variable sites; H, number of haplotypes; h, haplotype (genetic) diversity; π, nucleotide diversity; k, mean number of pairwise differences

Populations	Diversity indexes						
	n	bp	S	H	h	π	k
Arembepe	58	752	124	5	0.725 ± 0.028	0.05763 ± 0.00338	42.355
Praia do Forte	53	752	79	5	0.649 ± 0.048	0.04812 ± 0.00451	35.509
Sauípe	8	752	79	4	0.643 ± 0.184	0.02676 ± 0.01874	19.75
Overall	119	752	125	7	0.71 ± 0.027	0.05469 ± 0.00211	40.164
*Eim**	67	752	3	4	0.358 ± 0.069	0.00051 ± 0.00011	0.37992

Hybridization is of conservation concern especially when dealing with threatened species such as the hawksbill, since many studies report sterility or low fitness in hybrids (Allendorf et al. 2001). Nevertheless, hybrids can sometimes be viable, as is the case for females sampled in this study (nests are monitored until eggs hatch), so in this case, the hybridization might be accompanied by introgression, a process that implies the backcross of the hybrids with one or both parental taxa. The fact that most of the analyzed animals were undoubtedly diagnosed as hawksbills in the field argues in favor of this being a long term phenomenon, with F2 and later backcrosses. Considering the two distinct loggerhead haplotypes found, we could assume that at least two distinct events produced fertile F1 hybrids between male hawksbill and female loggerheads. However, these haplotypes are very common (about 75%) in the loggerhead population nesting in the same region (Soares 2004), thus recurrent hybridization cannot be discounted as an explanation for our results.

The breeding seasons of loggerhead, olive and hawksbill populations in Bahia overlap, although the nesting peak for hawksbill and loggerhead differ by a month. Olive ridleys are not commonly found in the study area, having a distribution biased to the extreme north of the state, where fewer hawksbills are encountered. Loggerheads in Bahia outnumber the hawksbills by a couple of thousand nests every year (Marcovaldi and Marcovaldi 1999). Besides, the studies by Marcovaldi et al. (1999) and Godfrey et al. (1999) reported that more than 90% of hawksbill hatchlings in Bahia are females. Thus, there is a strongly female-biased sex ratio. This, in addition to the much larger number of loggerheads that choose this area to breed, and the larger body size of female loggerheads as compared to the hawksbill male, makes it hard to understand why so many interspecific cross matings are happening. However, the high frequency of loggerhead haplotypes may be the result of the occurrence of F2 or further generation female hybrids backcrossing with hawksbill males and increasing the frequency the two loggerhead mtDNA types.

Even though gene flow is a normal evolutionary process, and genes and genotypes cannot be preserved unchanged, hybridization and introgression may threaten rare species existence (Rhymer and Simberloff 1996; Seehausen et al. 1997; Allendorf et al. 2001). Although hybridization between several Cheloniidae species has been described (Karl et al. 1995; Bowen and Karl 1996) studies with other hawksbill populations in the Caribbean (Bass et al. 1996; Díaz-Fernández et al. 1999) and Pacific (Broderick et al. 1994; Broderick and Moritz 1996; Okayama et al. 1996) nesting grounds did not report the occurrence of hybrids. Thus the unusually high (more than 40%) proportion of hybrids in the Brazilian population is apparently unique and should represent a serious conservation concern for Brazilian hawksbills, raising the polemic about conservation efforts focusing on hybrid populations (Allendorf et al. 2001).

The introgression process indicated by the putative backcrosses described here may be related to the population decline of both species in the recent past, although it can also be evidencing an ancient process, as suggested by Karl et al. (1995), given the long generation times of sea turtles and the long evolutionary history of these species, which appear during the Miocene or earlier. Thus, these may be the oldest species that naturally hybridize.

Whether this phenomenon represents or not a threat to the hawksbill population nesting in Brazil is hard to measure. The limited information provided by maternally inherited markers makes difficult to establish how many hybridization events and how long ago these events occurred. Further studies with nuclear markers are needed to better understand the implications and causes of such events, and its impact on the genetic diversity and identity of both species. Nuclear DNA analyses may also help to determine if loggerhead males are mating with hawksbill females as well. This information added to other ecological data can provide in the future important clues about the effect of anthropogenic pressures acting on sea turtles populations.

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