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Nuclear markers reveal a complex introgression pattern among marine turtle species on the Brazilian coast

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Abstract

Surprisingly, a high frequency of interspecific sea turtle hybrids has been previously recorded in a nesting site along a short stretch of the Brazilian coast. Mitochondrial DNA data indicated that as much as 43% of the females identified as *Eretmochelys imbricata* are hybrids in this area (Bahia State of Brazil). It is a remarkable find, because most of the nesting sites surveyed worldwide, including some in northern Brazil, presents no hybrids, and rare Caribbean sites present no more than 2% of hybrids. Thus, a detailed understanding of the hybridization process is needed to evaluate natural or anthropogenic causes of this regional phenomenon in Brazil, which could be an important factor affecting the conservation of this population. We analysed a set of 12 nuclear markers to investigate the pattern of hybridization involving three species of sea turtles: hawksbill (*E. imbricata*), loggerhead (*Caretta caretta*) and olive ridley (*Lepidochelys olivacea*). Our data indicate that most of the individuals in the crossings *L. olivacea* × *E. imbricata* and *L. olivacea* × *C. caretta* are F1 hybrids, whereas *C. caretta* × *E. imbricata* crossings present F1 and backcrosses with both parental species. In addition, the *C. caretta* × *E. imbricata* hybridization seems to be gender and species biased, and we also found one individual with evidence of multispecies hybridization among *C. caretta* × *E. imbricata* × *Chelonia mydas*. The overall results also indicate that hybridization in this area is a recent phenomenon, spanning at least two generations or ~40 years.

Keywords: *Caretta caretta*, *Chelonia mydas*, *Eretmochelys imbricata*, hybridization, introgression, *Lepidochelys olivacea*

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Introduction

The study of hybridization is important for understanding interspecies relationships and horizontal evolutionary processes (Allendorf *et al*. 2001; Seehausen 2004). However, the role of hybridization between species as part of a natural process of animal evolution is not well understood. Indeed, interspecific hybridization between well-recognized taxa is commonly considered as a deleterious process that can lead to extinction (Rhymer & Simberloff 1996). Interestingly, population decline and other consequences of human activities in the environment have been suggested as the most likely causes for the 'unnatural' appearance of interspecific hybrids (Allendorf *et al*. 2001). Anyway, hybridization can be also considered as part of the evolutionary history of many species of plants and animals. It is believed that 10% of animal species hybridize, and this proportion can reach as much as 100% in some groups of birds (Mallet 2005).

Molecular markers allow the study of hybridization events with greater precision than morphology-based

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approaches (Seminoff *et al.* 2003). The first molecular studies on hybridization used mitochondrial DNA (mtDNA) markers to characterize different parental species and screen for putative hybrids by searching mtDNA lineages from another species (Rhymer & Simberloff 1996). This approach also provides information on the gender of the parental species in the crossing that generates the F1 hybrids. However, the analysis of maternally inherited DNA alone does not provide a detailed picture on the extent of the hybridization or introgression processes. For example, if the F1 or >F1 hybrids are morphologically more similar or indistinguishable of the mitochondrial donor species, no significant information may be recovered from mtDNA analysis. Besides, in a hybrid swarm (i.e. where a high proportion of hybrids backcross with one parental species or another hybrid), the population will carry genes from both parental taxa but may be still morphologically indistinguishable, at least superficially, from one of the parental species. Finally, considering that a moderate influx of nuclear genes from one parental species into a population will lead to a significant proportion of hybrids to appear genetically pure, based on the analysis of few diagnostic loci (Rhymer & Simberloff 1996; Allendorf *et al.* 2001), the use of several unlinked segments of the genome will theoretically allow a better understanding of the current hybridization process. Thus, there is a need to analyse many biparentally inherited autosomal markers such as allozymes, microsatellites and single-nucleotide polymorphisms (SNPs).

Within the suborder Cryptodira (order Testudines), which includes most of extant tortoises and turtles, hybridization is fairly common within and between genera. Within the Geoemydidae family, some freshwater turtle species clearly show relatively weak reproductive isolation mechanisms (for a review see Buskirk *et al.* 2005), with 19 possible crosses between species being reported so far. Fritz *et al.* (2007) reported five hybrids of 72 individuals with mixed morphology between *Mauremys caspica* and *M. rivulata*, all reporting mtDNA of *M. caspica* and a variable combination of nuclear DNA alleles (C-mos and ISSR loci) for both parental species. In this study, the authors raised the possibility of a localized phenomenon resulting from secondary contact. Spinks and Shaffer (2007) studied species of the genus *Cuora*, known for having widespread hybridization due to natural and human-mediated causes and showed that *C. trifasciata* exhibited two highly divergent mtDNA clades, probably due to hybridization with *C. pani* or with *C. aurocapitata*.

Within the genus *Chelonoidis* of the Testudinidae family, Farias *et al.* (2007) analysed two species from the Amazon forest and found four hybrids with mixed morphology between *C. carbonaria* and *C. denticulata*, all with *C. denticulata* mtDNA. In this case, both species are sympatric, but the higher density and current expansion of *C. denticulata* may be the likely cause of hybridization.

Within sea turtles (family Cheloniidae), hybridization events have long been recognized, although scientific reports are still scarce. Most studies involving sea turtle hybridization were based solely on the description of individuals with intermediate morphological characters (Carr & Dodd 1983; Kamezaki 1983; Wood *et al.* 1983; Frazier 1988), and only recently, have these hybridization events been investigated with molecular markers (Conceição *et al.* 1990; Karl *et al.* 1995; Seminoff *et al.* 2003; Lara-Ruiz *et al.* 2006; Reis *et al.* 2010a). Since the first description of a marine turtle hybrid by Garman in 1888 (Karl *et al.* 1995), many interspecific hybrids have been investigated using molecular markers. These studies include crossings between green turtles (*Chelonia mydas*) and hawksbills (*Eretmochelys imbricata*) (Wood *et al.* 1983; Karl *et al.* 1995; Seminoff *et al.* 2003), loggerheads (*Caretta caretta*) and *E. imbricata* (Kamezaki 1983; Frazier 1988; Lara-Ruiz *et al.* 2006), *C. caretta* and olive ridleys (*Lepidochelys olivacea*) (Karl *et al.* 1995; Reis *et al.* 2010a), *C. mydas* and *C. caretta* (Karl *et al.* 1995), and *E. imbricata* and *L. olivacea* (Lara-Ruiz *et al.* 2006). It is remarkable that sea turtle hybrids are observed between species belonging to tribes Carettini (*L. olivacea*, *C. caretta* and *E. imbricata*) and Chelonini (*C. mydas*), whose recent phylogenetic evidence indicates a deep time divergence of about 63 million years ago (Naro-Maciel *et al.* 2008).

The current study focuses on the hybridization among sea turtle species on the Brazilian coast, where an atypically high frequency of interspecific hybrids have been documented (Lara-Ruiz *et al.* 2006; Reis *et al.* 2010a). Previous studies found different patterns and rates of introgression occurring in this restricted region of the Brazilian coast, between Bahia and Sergipe States. Karl *et al.* (1995) investigated four hatchling clutchmates and found a combined profile of mtDNA and nuclear restriction fragment length polymorphism (RFLP) markers that indicated these hatchlings were F1 hybrids between a *C. caretta* female and a *C. mydas* male. Bass *et al.* (1996) showed that 10 of 14 morphologically identified *E. imbricata* females from a nesting population in the Bahia State carried *C. caretta* mtDNA haplotypes. Recently, Lara-Ruiz *et al.* (2006) showed in a large survey of *E. imbricata* turtles (n = 117) nesting in Bahia that at least 43% of females were hybrids, because individuals morphologically identified as *E. imbricata* presented *C. caretta* mtDNA haplotypes and, on a much smaller scale, *L. olivacea* haplotypes. These data also suggested a gender and species biased mating because no *C. caretta* or *L. olivacea* individuals (as identified by morphology) bearing *E. imbricata*

mtDNA haplotypes have been identified so far. Further, several viable nests have been verified for the hybrid females in Bahia (Lara-Ruiz *et al.* 2006), raising the possibility of an ongoing introgression process in the Brazilian coast due to the fertility of female F1 hybrids. Another recent publication (Reis *et al.* 2010a) investigated the hybridization between *C. caretta* and *L. olivacea* observed in Sergipe State and found that among 51 *C. caretta* individuals analysed, 14 (27.5%) exhibited *L. olivacea* mtDNA. In this study, a few hybrids were found to present mixed morphological characters, which were suggested to be likely generated by introgression.

To evaluate in detail the hybridization phenomenon observed among sea turtles in Brazil, we analysed 12 autosomal markers in nesting and feeding sites containing both hybrid individuals and 'pure' parental species. We used previously described RFLPs and microsatellites and developed new haplotype markers from autosomal gene sequences to be used as diagnostic characters for *C. caretta*, *E. imbricata*, *C. mydas* and *L. olivacea*. Our goals here are to use all available data to assess hybridization among sea turtles observed off the coast of Brazil to investigate important conservation issues and discuss some management implications.

Methods

Sampling

We obtained a total of 387 samples. Among these, we sampled 320 'pure' individuals of the four Cheloniidae species nesting in Brazil: 168 *C. caretta*, 121 *E. imbricata*, 22 *L. olivacea* and nine *C. mydas* (Fig. 1). All of these individuals displayed morphology and mtDNA of the respective species. Except for the nesting sites in Bahia and Sergipe coastlines, no hybrid was previously registered among nesting and bycatch individuals from the sampling sites (Fig. 1).

Of these 387 samples, 66 individuals previously identified as hybrids (morphology of one species and mtDNA from a different one, as described in the Introduction section) were analysed with nuclear markers, and those included 50 hybrids of *C. caretta* × *E. imbricata* and two hybrids of *E. imbricata* × *L. olivacea* analysed by Lara-Ruiz *et al.* (2006) and 14 hybrids of *L. olivacea* × *C. caretta* analysed by Reis *et al.* (2010a). Among the *C. caretta* × *E. imbricata* hybrids, we used DNA samples of four siblings derived from a single clutch (R0264, R0265, R0267, and R0268) in particular analyses. These four samples were collected in Praia do Forte, Bahia, and possessed *C. caretta* mitochondria (Lara-Ruiz *et al.* 2006), but the morphology indicated a possible hybridization between *E. imbricata* and

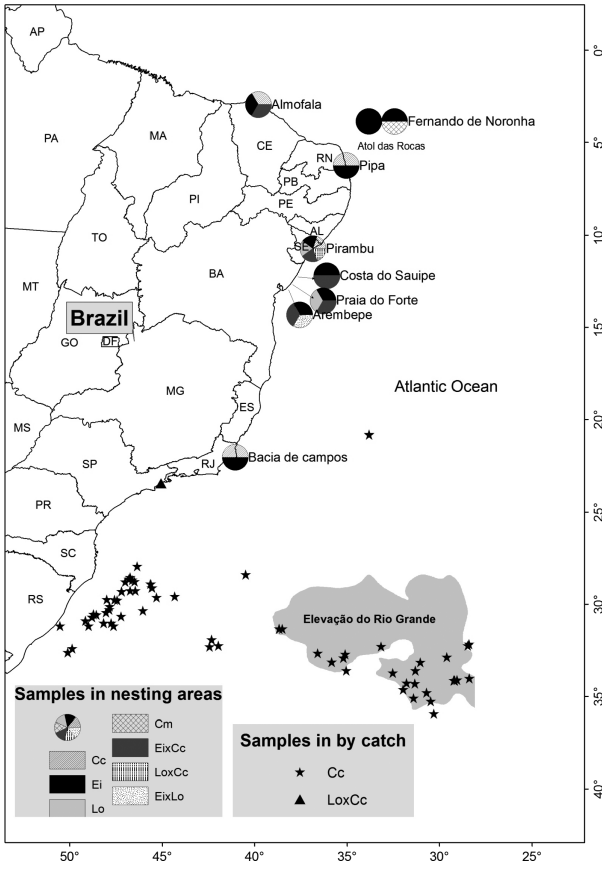

Fig. 1 Map displaying the sampling locations along the Brazilian coast. Circles do not refer to sample proportions, but represent the species or hybrid class samples found in each area. Cc, *Caretta caretta*; Ei, *Eretmochelys imbricata*; Lo, *Lepidochelys olivacea*; Cm, *Chelonia mydas*.

C. mydas. Another sample used in this study included one hatchling (R0025) of a *C. caretta* × *E. imbricata* hybrid female (R0024) that was previously analysed by Lara-Ruiz *et al.* (2006). Besides the four siblings from a single clutch and the hatchling R0025, all other hybrid samples were adult nesting females.

We have also included one bycatch sample (R0384) that was previously classified by morphology as *C. caretta*, but identified here by mtDNA as a *L. olivacea* × *C. caretta* hybrid from the São Paulo State (unpublished data).

Definition of hybridization and introgression

Throughout this study, we use the terms hybridization and introgression as defined by Rhymer & Simberloff (1996). According to these authors, hybridization is defined as 'interbreeding of individuals from what are believed to be genetically distinct populations, regardless of the taxonomic status of such populations', while

introgression can be described as 'gene flow between populations whose individuals hybridize, achieved when hybrids backcross to one or both parental populations'. In the case of this study where molecular markers were used to investigate the introgression (Fig. S4, Supporting information), F1 hybrids exhibited for all loci two alleles derived from different species (e.g. a *C. caretta* × *E. imbricata* F1 hybrid shows for all loci one allele of *C. caretta* and one of *E. imbricata*), while introgression (>F1) is considered when, for one sample, one or more loci exhibit both alleles of only one species (e.g. for RAG1 both alleles are from *C. caretta*, regardless of whether they are the same allele or two different private *C. caretta* alleles). In this situation, we define the presence of two alleles derived from the same species as homospecific, even if they are different allele states. In the case of microsatellites, due to the high number of shared alleles, introgression was only considered for a previously known hybrid (from mtDNA, morphology and/or nuclear sequences), and when both alleles of a locus were exclusive (private) alleles of a single species.

The introgression can also be subdivided in two different processes: unidirectional (an event when an F1 hybrid backcrosses with one parental population only) or as bidirectional (when hybrids can backcross with either parental species). To be considered as a bidirectional introgression, a sample needs to exhibit at least two different loci combinations of introgression (e.g. RAG1 with both private alleles of *E. imbricata* and R35 with two private alleles of *C. caretta*).

Nuclear genotype determination

For an initial screening on *E. imbricata* and *C. caretta* hybrids, we analysed three anonymous autosomal sequences (CM-12, CM-14 and CM-28) through PCR-RFLP with primers previously developed for *C. mydas* (Karl & Avise 1993) that presented species-specific profiles for *E. imbricata* and *C. caretta* (Karl & Avise 1993; Karl *et al.* 1995).

We genotyped four autosomal microsatellites including OR1 and OR3 (Aggarwal *et al.* 2004), and Cc1G02 and Cc1G03 (Shamblin *et al.* 2007), which were developed for *L. olivacea* and *C. caretta*, respectively. All genotypes were evaluated to determine species-specific alleles. A smaller database was used (as described later) for further analysis. Details on laboratory methods for each marker can be found on supporting information (Appendix S1, Supporting information).

We used published nuclear markers (Krenz *et al.* 2005; Le *et al.* 2006; Naro-Maciel *et al.* 2008) to evaluate the presence of interspecific variation in four exons [brain-derived neurotrophic factor (BDNF), oocyte maturation factor (Mos – Cmos), two recombination activating genes (RAG1 and RAG2)] and one intron [RNA fingerprint protein 35 gene (R35)]. Once the most variable regions among species were identified for each locus, we synthesized flanking primers to amplify short target segments (amplicons of ~500 bp), allowing to perform a fast genotyping of interspecific variation. Primer sequences are shown in Table S1 (Supporting information). For the five nuclear loci analysed, sequences were first generated from 'pure' parental-type individuals of the species coming from areas where no hybrids have been recorded (see Sampling section, and Fig. 1). The gametic phases of all heterozygote sequences were reconstructed using PHASE (Stephens & Donnelly 2003).

Introgression analysis

To identify the private (species-specific) alleles, we used all samples typed for each species (complete data set), but for the introgression analysis, we only used individuals with at least seven loci typed (filtered data set, maximum of two loci with missing data considering both microsatellites and sequences), totalling 223 individuals (Tables S2 and S3, Supporting information). The only two exceptions are one hybrid of *E. imbricata* × *L. olivacea* and one *C. mydas* individual, which displayed three missing loci data each sample.

Bayesian clustering methods were used to detect the level of introgression combining the microsatellite and sequence data set using the programs STRUCTURE (Pritchard *et al.* 2000), NewHybrids (Anderson & Thompson 2002), and HybridLab (Nielsen *et al.* 2006). Nuclear sequences were coded as haplotypes. The RFLPs were only used for an initial screening of *E. imbricata* and *C. caretta* hybrids, because the three loci used do not identify other species besides these two. For this reason, the RFLP loci were excluded from the Bayesian analyses. For STRUCTURE, five independent runs for each value of *K* (number of populations) from 1 to 10 were performed at 1 000 000 Markov Chain Monte Carlo (MCMC) repetitions with a 100 000 burn-in period assuming uncorrelated allele frequencies and admixture. The best *K* was assessed using Evanno's methodology (Evanno *et al.* 2005) using the online tool Structure Harvester (Earl & VonHoldt 2012). All figures from STRUCTURE output were generated with Distruct (Rosenberg 2004). For NewHybrids, six classes were considered for identification, because no hybrids beyond F2 can be statistically detected (Anderson & Thompson 2002). Three independent runs were performed with 10 000 burn-in steps and 1 000 000 steps after the sweep. The four individuals that were found in the same nest were excluded, because they exhibited the haplotype combination of three species, a scenario that

NewHybrids is not designed to detect; NewHybrids uses a model that considers only two hybridizing species. No prior information on parental species was used in this analysis. To be considered as part of a class (pure parental, F1, F2, or backcross), a probability of at least 0.9 and 0.75 had to be assigned to an individual in STRUCTURE and NewHybrids, respectively. To test whether the markers had enough resolution to distinguish among parental species, F1, F2 and backcrosses, we simulated five data sets in HybridLab, combining the parental samples from *E. imbricata* and *C. caretta* to simulate *E. imbricata* × *C. caretta* hybrids and their following hybridization events (backcrosses and F2). *Lepidochelys olivacea* and *C. caretta* parental samples were combined in the same way to simulate *L. olivacea* × *C. caretta* hybrids. All 10 simulated data sets were analysed in STRUCTURE and NewHybrids. To explore graphically the distribution of genetic variation between individuals, the software Genetix (Belkhir *et al.* 1996–2004) was used to produce a 3D graph of factorial correspondence analysis (FCA) using both microsatellite and sequence data. To depict the relationship among haplotypes, a genealogical network was constructed using Network v4.61 (fluxus-engineering.com) using the median-joining algorithm (Bandelt *et al.* 1999). Finally, to determine whether the microsatellite markers could recover the same information as the combined data set (microsatellites and sequences), we also estimated the level of admixture using the software STRUCTURE. All simulations were run as described previously.

Results

PCR-RFLPs

Of the 50 hybrids of *E. imbricata* and *C. caretta* analysed, 42 were typed for all three RFLP loci. No intraspecific polymorphism was identified and each species presented a single exclusive genotype, which made the identification of hybrids straightforward. Four individuals displayed homospecific alleles (both derived from a single parental species) of *E. imbricata* for at least one of three loci, including the hatchling of a nesting *E. imbricata* × *C. caretta* hybrid female. No individual was homospecific for *C. caretta* alleles. Among eight hybrid samples that were typed for only one or two loci, five were also homospecific for *E. imbricata* alleles and four of them belonged to the clutch samples. In total, nine of 50 hybrids displayed introgression with *E. imbricata*, and the remaining 41 individuals showed alleles of both species in all loci analysed, indicating that they are likely F1 hybrids. Using these three RFLP loci, no hybrids were detected among the 121 'pure' individuals bearing *E. imbricata* mtDNA and morphology, or among a subset of 40 individuals identified as *C. caretta* by morphology and mtDNA.

Autosomal haplotypes detected by sequencing

The number of haplotypes and polymorphisms identified in each gene segment is shown in Table 1. Details of the haplotype sequences identified in each individual are listed in Table S3 (Supporting information). Except for two RAG1 haplotypes shared between *E. imbricata* and *L. olivacea* (which were found in low frequency among the hybrids) and one BDNF haplotype fixed in both *E. imbricata* and *C. caretta*, all haplotypes were species-specific, thereby enabling a detailed analysis of hybridization and introgression. The Cmos gene could not be amplified in our *C. mydas* samples; therefore, we used the published sequences from Naro-Maciel *et al.* (2008) to analyse this locus. The R35 intron contained the most variation, while BNDF was relatively invariant and did not show any differences between *E. imbricata* and

Table 1 Results for the sequenced autosomal haplotypes

Locus	Size (bp)	Polymorphic sites	Number of haplotypes	Singletons	Parsimony informative sites	Exclusive haplotypes			
						Ei	Cc	Lo	Cm
RAG2	620	6 (6)	6 (6)	3 (0)	3 (6)	2 (2)	1 (1)	2 (2)	1 (1)
BDNF	559	6 (7)	5 (6)	3 (2)	3 (5)	—	—	1 (1)	3 (4)
Cmos*	602	13 (20)	11 (14)	3 (4)	10 (16)	5 (6)	4 (4)	2 (2)	(3)*
R35	439	12 (14)	12 (14)	5 (3)	7 (11)	8 (8)	2 (2)	1 (1)	2 (4)
RAG1	468	10 (10)	9 (10)	4 (1)	5 (9)	1 (1)	2 (2)	4 (4)	1 (1)
Total	2688	46 (57)	44 (51)	18 (10)	28 (47)	15 (17)	9 (9)	10 (10)	7 (13)

*For Cmos, no *C. mydas* sequence was obtained in this study, so we have used only the ones published elsewhere (Naro-Maciel *et al.* 2008).

The data presented in brackets also consider sequences of *E. imbricata*, *C. caretta*, *L. olivacea*, and *C. mydas* published elsewhere (Naro-Maciel *et al.* 2008) combined with the sequences obtained in this study.

C. caretta. The genealogical relationships (median-joining networks) between haplotypes for the five autosomal segments are shown in Fig. S1 (Supporting information).

Among the 50 *C. caretta* × *E. imbricata* hybrids (morphologically identified as *E. imbricata*, but having a *C. caretta* mtDNA), 16 showed a sign of introgression with either RFLP, sequences or microsatellites. We found evidence of introgression with *E. imbricata* and in a smaller frequency with *C. caretta* (Table 3).

The other interspecific hybrids, *L. olivacea* × *E. imbricata*, and *L. olivacea* × *C. caretta* exhibited a typical pattern (one private allele from each parental species) of F1 hybrids for all loci, except for one *L. olivacea* × *C. caretta* sample (# 882) which presented two *L. olivacea* alleles in the BDNF gene (Table S3, Supporting information).

Interestingly, we found two hybrids among the samples with no prior evidence of hybridization. One *E. imbricata* sample classified by morphology and mtDNA as *E. imbricata* (R0069) showed both *C. caretta* and *E. imbricata* alleles. For this sample, we could observe a hybrid pattern in three loci (RAG1, Cmos and RAG2) and for R35, two *E. imbricata* alleles were observed. This sample comes from a feeding area where no hybrids have been reported (Atol das Rocas, Rio Grande do Norte State). The mitochondrial haplotype found matches the EATL haplotype found by Bowen *et al.* (2007) and is more related to Indo-Pacific (Australian) haplotypes; thus, this hybrid is likely derived from a nesting site outside the Brazilian coast. For another sample, classified as *C. caretta* by morphology (R0384) and sampled along the São Paulo coastline, we have also detected a *L. olivacea* mtDNA haplotype (control region haplotype F). This sample exhibited *L. olivacea* and *C caretta* alleles in all nuclear loci analysed and is thus considered here a F1 hybrid. Interestingly, it was captured in an area (Ubatuba, São Paulo) where *C. caretta* and *L. olivacea* are not usually found.

In addition, we analysed in detail the offspring consisting of four putative hybrid individuals from the same nest (R0264, R0265, R0267 and R0268). They present interesting allele combinations of the species *E. imbricata*, *C. caretta* and *C. mydas* (Table S3, Supporting information), including a clear sign of homospecific alleles for *E. imbricata* in individual R0267 (Table 3).

The analysed hybrid female and its hatchling (R0024 and R0025, respectively) presented an interesting scenario, because the female is a F1 hybrid, but the hatchling showed a clear introgression pattern with *E. imbricata* for Cmos and RAG2 genes.

Microsatellites

For the four loci analysed, it was possible to find some species-specific alleles (Table 2). For *E. imbricata*

Table 2 Results for microsatellites

	n	A	Alleles/locus	Allelic range (bp)				Exclusive alleles	Shared alleles	H_o	H_e
				OR1	OR3	Cc1G02	Cc1G03				
Caretta caretta	71 (169)	34 (43)	8.5 (10.75)	156 (148–160)	139–163 (139–163)	262–318 (262–326)	271–335 (271–335)	25	18	0.55 (0.44)	0.62 (0.50)
Eretmochelys imbricata	76 (121)	18 (20)	4.5 (5.0)	156–168 (156–168)	159–185 (159–185)	278–330 (278–330)	267–283 (259–283)	4	16	0.25 (0.26)	0.30 (0.32)
Lepidochelys olivacea	16 (22)	19 (20)	4.75 (5.0)	168–228 (164–228)	143–163 (143–163)	286–330 (286–330)	271–299 (271–299)	9	11	0.50 (0.51)	0.51 (0.51)
Chelonia mydas	3 (3)	5 (5)	1.67 (1.67)	160 (160)	171–199 (171–199)	–	271 (271)	3	2	0.50 (0.50)	0.83 (0.83)
C. caretta × *E. imbricata*	50 (55)	30 (31)	7.5 (7.75)	156–168 (156–168)	159–185 (159–161)	262–326 (262–326)	267–331 (275–331)			0.87 (0.87)	0.68 (0.68)
L. olivacea × *E. imbricata*	2 (2)	6 (6)	2.0 (2.0)	164–168 (164–168)	159–163 (159–163)	–	267–271 (267–271)			1.00 (1.00)	0.67 (0.67)
L. olivacea × *C. caretta*	13 (14)	26 (26)	6.5 (6.5)	156–208 (156–208)	161–163 (161–163)	270–330 (270–330)	271–327 (271–327)			1.00 (1.00)	0.73 (0.72)

n, Number of samples genotyped; A, number of alleles observed; H_o, observed heterozygosity; H_e, expected heterozygosity. Numbers in brackets are derived from the complete data set. Exclusive alleles refer to alleles found in one species, while shared alleles are found in two or more species.

samples, only four exclusive alleles were found, probably because most of the allele size range was shared with *C. caretta* and *L. olivacea*. The observed and expected heterozygosity values are reported in Table 2. The high *Ho* value for all hybrid populations clearly suggests that the majority of analysed hybrids are F1 (Table 2). Only three individuals could be assigned as introgressed: (i) a *C. caretta* × *E. imbricata* hybrid exhibited introgression with *C. caretta* (R0080); (ii) an individual from a feeding area (Atol das Rocas) identified morphologically as *E. imbricata* showed a signal of hybridization with *C. caretta* followed by introgression with *E. imbricata* (R0069); and (iii) one individual (R0267) of the nest of four offspring that also presents *E. imbricata* introgression.

Bayesian introgression analyses using microsatellites and sequences

Bayesian clustering analysis performed in STRUCTURE using only microsatellite genotypes retrieved a best estimated $K = 4$ although two peaks can be seen in the values of ΔK, one in $K = 2$ and the other in $K = 4$ (Fig. S2, Supporting information). We could differentiate *E. imbricata*, *C. caretta*, *L. olivacea* and the fourth group was composed by *E. imbricata* × *C. caretta* hybrids (Fig. S5, Supporting information). However, most of the *C. caretta* and *L. olivacea* parental individuals showed a sign of admixture probably due to the presence of shared alleles, and the *C. caretta* × *L. olivacea* hybrids exhibited a much higher component of *L. olivacea* than observed with the combined analysis with sequences (see below). Thus, to estimate with higher precision the introgression pattern of an individual, SNPs or sequences should be added to the analyses because they have a higher proportion of exclusive alleles and much lower homoplasy level than microsatellites.

The combined analyses of microsatellites and sequences generated similar results in STRUCTURE and NewHybrids. The best K retrieved from STRUCTURE was equal to 3 (Fig. S2, Supporting information), separating the species *E. imbricata*, *C. caretta* and *L. olivacea* in different groups (Fig. 2). However, *Chelonia mydas* and *L. olivacea* were incorrectly grouped due to the low sampling size and occurrence of many shared alleles in microsatellite loci. Thus, using $K = 4$, *C. mydas* can be differentiated from *L. olivacea*, as well as hybrids possessing *C. mydas* alleles (Fig. 2). According to the FCA results (Fig. S3, Supporting information), all hybrids appear intermediary regarding their parental species.

Regarding the hybrid classes, all *L. olivacea* × *E. imbricata* and *L. olivacea* × *C. caretta* exhibited equal components of each parental species (F1). The *E. imbricata* × *C. caretta* hybrids displayed different degrees of hybridization and introgression with each parental species (Fig. 2). The hybrid offspring of four individuals exhibited different components of introgression (Fig. 2). One individual classified as a 'pure' *E. imbricata* (R0069) showed also *C. caretta* alleles.

In the analysis with the NewHybrids programme, all pure individuals of each parental species could be correctly assigned to its respective class ($P > 0.98$). For the *L. olivacea* × *C. caretta* hybrids ($n = 14$), NewHybrids analysis (Fig. 3) indicated that they are all F1 ($P > 0.81$). Sample #882 (a putative *L. olivacea* × *C. caretta* hybrid) was classified as introgressed but with lower probability ($P = 0.81$), while the others showed a probability of 0.99 of being F1 hybrids. For *C. caretta* × *E. imbricata* hybrids ($n = 46$, excluding four hatchlings), 40 individuals were classified as F1 ($P > 0.75$), 37 had a probability higher than 0.95 (Fig. 3). Among the remaining six samples, two were classified as backcrosses with *E. imbricata* ($P > 0.98$), one as backcross with *C. caretta* ($P = 0.78$), and three were not classified with confidence to a specific class. Of those which were not classified with confidence, two (R0078 and R0217) had low probability ($P < 0.01$) of belonging to either parental class; thus, these individuals have a posterior probability of

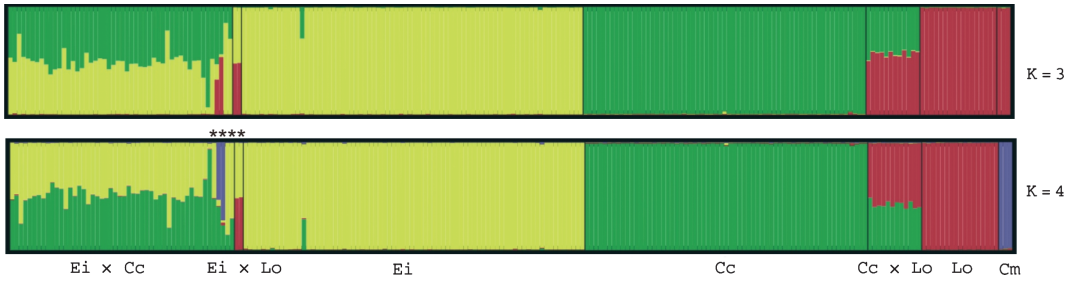

Fig. 2 Output graphic from the introgression analysis (admixture) in the program STRUCTURE with nine nuclear markers (five autosomal sequences and four microsatellites). The *x*-axis represents each individual being analysed, and the *y*-axis depicts the estimated admixture proportions related to each parental species. Asterisks depict the four hybrid offspring of a clutch. Abbreviations and species colour codes: Cc, *Caretta caretta* (green); Ei, *Eretmochelys imbricata* (yellow); Lo, *Lepidochelys olivacea* (red); Cm, *Chelonia mydas* (blue).

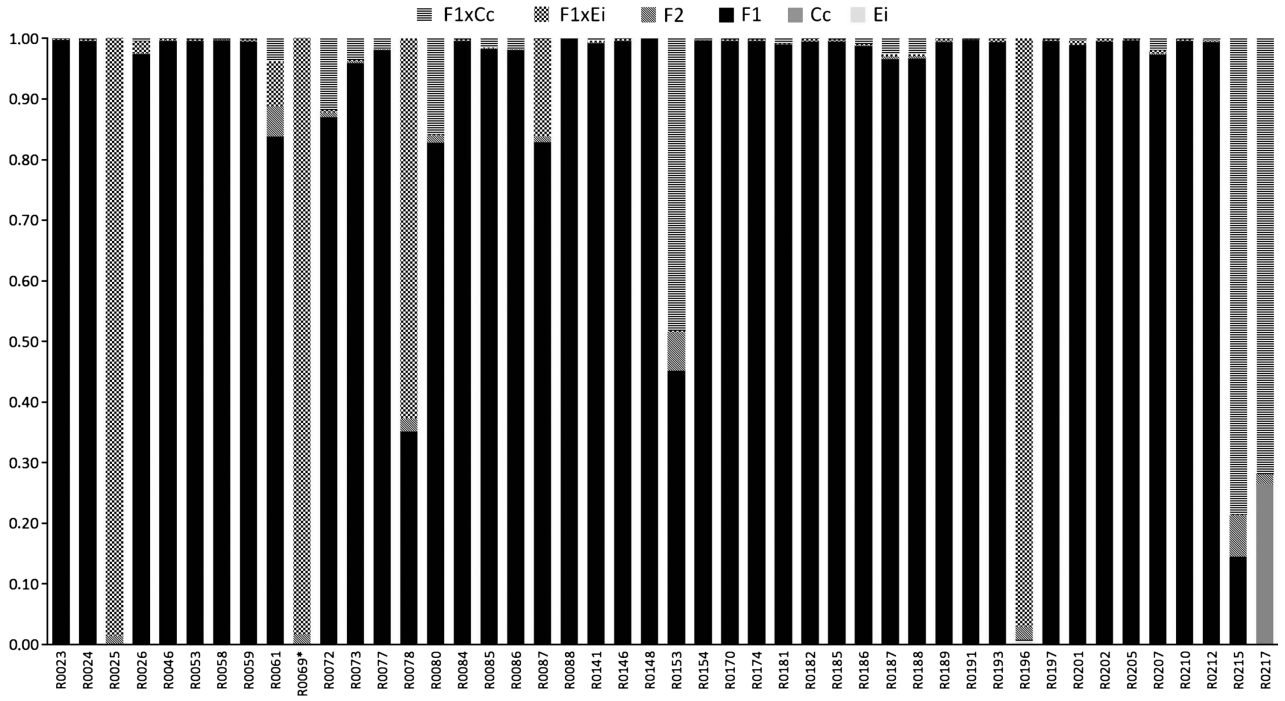

Fig. 3 Graph showing the results from NewHybrids for all *C. caretta* × *E. imbricata* hybrids and the *E. imbricata* individual found in a foraging area (Atol das Rocas) identified as a hybrid (marked with an asterisk). The *Y*-axis is the posterior probability of each sea turtle being pure (*E. imbricata* or *C. caretta*), F1 or F2 hybrid, or backcrossed with one of the parental species.

Table 3 Summary of introgressed individuals ($n = 17$) obtained with autosomal RFLPs, sequences and microsatellite markers. Each column represents the loci showing a sign of introgression (homospecific alleles). Circle represent introgression with *L. olivacea*, triangles with *C. caretta*, and *X*'s with *E. imbricata*. Sample codes refer to individual identification

	RFLP*			Sequence					Microsatellites
Sample code	CM28	CM14	CM12	RAG1	RAG2	CMOS	BDNF	R35	CC1G03
R0025	X				X	X			
R0061						X			
R0069								X	
R0078				X					
R0080									Δ
R0084			X						
R0088			X						
R0153				Δ					
R0177				X					
R0196	X	X			X	X			
R0217			X		Δ	Δ			
R0260		X							
R0264		X				Δ			
R0265		X							
R0267				X	X	Δ			X
R0268		X							
#882							○		

*As RFLP loci do not detect *C. mydas* alleles, the results for individuals from a clutch (R0264, R0265, and R0268) should be seen with caution, as they may not exhibit true introgression with *E. imbricata* for these loci.

>0.99 for being hybrids of some sort, while the third one (R0153) has a clear signal of introgression with *C. caretta* in one nuclear locus (Table 3) and exhibited almost the same probability of belonging either to *C. caretta* or to a backcross F1 × *C. caretta*. The *E. imbricata* sample from Atol das Rocas previously classified

as a 'pure' individual by morphology and mtDNA (R0069) exhibited *C. caretta* alleles in three nuclear loci and presented a probability of 0.98 of being a backcross with *E. imbricata*. No individual was classified as F2 that could be generated by the product of two F1 hybrids.

For the simulated data from HybridLab, STRUCTURE correctly assigned each class. In the NewHybrids analysis with *L. olivacea* × *C. caretta* hybrids, the highest rate of misassignment was observed in F2 hybrids, 28% were assigned in either a different class or were assigned with low probability, only 3% of F1 hybrids and 18% of the backcrosses were assigned to a different class or were assigned with low probability. All parental individuals had a probability of >0.98 to belong to their class. For the *C. caretta* × *E. imbricata* hybrids, the highest rate of misassignment was also seen for F2 hybrids (27%), and the parental individuals were all assigned to their respective class with probability of >0.98, while 2% of F1 hybrids and 15% of the backcrosses were assigned to a different class or were assigned with low probability.

Population structure

For the autosomal sequence markers RAG2, BDNF, CMOS and R35, no structure was observed within species. However, RAG1 clearly shows some level of spatial differentiation in *C. caretta* populations (Fig. S1, Supporting information). The samples collected from foraging aggregations and rookeries differ in their RAG1 haplotype composition. *Caretta caretta* samples from bycatches ($n = 67$) showed only one haplotype (Hap2), which is identical to the haplotype found in both Atlantic and Pacific oceans by Naro-Maciel *et al.* (2008). As for the rookeries from Rio de Janeiro and Sergipe States (Reis *et al.* 2010b), in addition to Hap2, we found another typical haplotype (Hap6), which is present in *C. caretta* × *L. olivacea* hybrids. Interestingly, the *C. caretta* × *E. imbricata* hybrids from nesting populations in Bahia also present only Hap2, suggesting that *C. caretta* individuals that are mating either with *L. olivacea* or with *E. imbricata* in different Brazilian regions may come from different gene pools.

Discussion

A previous study (Lara-Ruiz *et al.* 2006) showed that morphologically identified *E. imbricata* female turtles exhibited mtDNA haplotypes characteristic of *C. caretta*. The same hybrid population was also analysed with nuclear markers in this study and showed that the individuals with *C. caretta* mtDNA and *E. imbricata* morphology exhibited alleles of both species. However, the pattern of introgression obtained from different markers varied remarkably for *E. imbricata* × *C. caretta* hybrids. Although the RFLP analysis matched a pattern of unidirectional backcross with one parental species (*E. imbricata*), autosomal sequences and microsatellites revealed introgression with *C. caretta* (Figs 2 and 3). The overall results suggest that the primary matings that generate F1 hybrids happen between a *C. caretta* female and an *E. imbricata* male (Fig. S4, Supporting information). The female F1 can then mate with either parental species, because our nuclear data revealed introgression with *E. imbricata* and *C. caretta* (Fig. S4, Supporting information). If hybrid males are also fertile, the mating with *E. imbricata* females is not frequent, because only one hybrid from a foraging area (Atol da Rocas) showed an *E. imbricata* mtDNA haplotype, but it is most likely not derived from the Brazilian rookeries.

Another interesting clue comes from the clutch of four hybrid siblings. In the offspring showing *C. caretta* mtDNA, we found autosomal alleles of *E. imbricata*, *C. caretta* and *Chelonia mydas*. Thus, using nuclear sequences, we could identify this new hybrid class (*C. caretta* × *E. imbricata* × *C. mydas*), which was previously classified as *C. caretta* × *E. imbricata* hybrid using only mtDNA (Lara-Ruiz *et al.* 2006). Considering the combination of genotypes present in the offspring, we hypothesize that a F1 *C. caretta* × *E. imbricata* female copulated with at least two males including: a *C. mydas* (evidenced by the R0264 and R0265 offspring genotypes) and an *E. imbricata* (R0267 offspring genotype). Indeed, it is well known that multiple paternities are common among sea turtles (Pearse & Avise 2001; Lee *et al.* 2004; Zbinden *et al.* 2007; Uller & Olsson 2008). If true, then it is possible that hybrid females may be more predisposed to mate with males of different species than ''pure'' females. The fourth individual (R0268) holds an ambiguous genotype, for which a likely mating pattern cannot be drawn (for details, see Fig. S4 in Supporting information). Although mating between two hybrids (*E. imbricata* × *C. caretta* and *E. imbricata* × *C. mydas*) could generate this clutch, the former hypothesis seems more plausible because no *E. imbricata* × *C. mydas* hybrids have been reported in this nesting area.

Among *L. olivacea* × *C. caretta* hybrids, we observed that the mating occurred in either direction, as we detected hybrids with mitochondria of either parental species. We found that the crossings between a female *L. olivacea* with a male *C. caretta* are more common, because 14 samples were found with *L. olivacea* mtDNA, while only one showed a *C. caretta* mtDNA. Most of the individuals were identified as F1 hybrids; thus, some sort of reproductive barrier might be present affecting the fertility of the progeny.

For *L. olivacea* × *E. imbricata* hybrids, only matings between a female *L. olivacea* and an *E. imbricata* male were observed, because the two identified hybrids had *L. olivacea* mitochondria. These individuals showed no sign of introgression; therefore, they might be infertile or could be the progeny of a rare hybridization event.

As most *C. caretta* × *E. imbricata* hybrids were also characterized as F1, we suggest that hybridization may be a relatively recent phenomenon happening on the Brazilian coast and/or the F1 hybrids may be less fertile than 'pure' *E. imbricata* or *C. caretta* individuals. The observation of hybrids introgressed with either parental species raises the possibility that hybrid individuals could display behavioural differences which could lead to divergent mating preferences, some tending to mate with *E. imbricata* and others with *C. caretta*. This behavioural difference was already observed among some of these hybrids found along the Brazilian coast where individuals exhibited different pattern of migrations in relation to either parental species (Marcovaldi *et al.* 2012, see details below). However, most introgression is observed among *E. imbricata*, which could be attributable to a decreased success of backcrossing with *C. caretta* either due to the low mating success of *C. caretta* males with female hybrids or to the low survival/fecundity of the backcrossed progeny with *C. caretta*. Furthermore, a single individual with apparent introgression with both parental species (R0217) could be either produced in three generations (F1 hybrid × *E. imbricata* × *C. caretta*) or two generations in a progeny of two F1 hybrids.

In the most common crossings (Fig. S4, Supporting information), females of *C. caretta* mate with males of *E. imbricata* or *L. olivacea* that are smaller than *C. caretta* males, therefore making it difficult to claim that body size may have an important role. Furthermore, a hypothesis raised by Karl *et al.* (1995) suggesting the tendency of the female parent to be smaller than the male in sea turtle hybridization was not supported in our study. Among all interspecific crossings (Fig. S4), except in the uncommon mating *L. olivacea* female × *C. caretta* male, the male is expected to be smaller than the female parent.

We observed that the common species (*C. caretta*) provide the female parent in the most common hybrid crossings, *E. imbricata* × *C. caretta* and *L. olivacea* × *C. caretta*. This observation is opposite to the pattern seen in most of the other animals (Wirtz 1999; Vianna *et al.* 2006). Thus, the tendency for the rare species to be the female in a hybrid cross as indicated by Karl *et al.* (1995) is not seen in most of the crossings observed in this study. However, sea turtle males are well known for displaying promiscuous mating behaviour (Karl *et al.* 1995; Lee *et al.* 2004), which may partially explain why the common species are most often the parental females. In any event, sex ratio differences between species and relative abundance of males and females from each species along the coastline during reproductive seasons could also explain the biased occurrence of hybrids, but no evidence is available.

Another fact to consider is related to the timing of reproductive seasons of the parental species in the region. In Bahia, the nesting seasons of *E. imbricata* and *C. caretta* slightly overlap, with peaks from 15 October to 15 December for *C. caretta* and from 15 December to 15 February for *E. imbricata* (Marcovaldi *et al.* 1999). By the time, the reproductive peak of *C. caretta* females is finishing, *E. imbricata* peak is starting; thus, *E. imbricata* males would be expected to be at coastal waters close to the nesting beaches. The slight overlap of nesting seasons and the higher abundance of *C. caretta* in the Brazilian coastline could cause *E. imbricata* males to encounter higher number of *C. caretta* females facilitating the observed gender biased hybridization. Following the same reasoning, the possibility of encounter between *E. imbricata* females and *C. caretta* males or F1 female hybrids (if they behave as *E. imbricata*) would be lower because *C. caretta* males will leave the Brazilian coast before *E. imbricata* females arrive to the nesting beaches, making the introgression with *C. caretta* unlikely. Unfortunately, there are no data available on the abundance of sea turtle males along the Brazilian coastline during nesting seasons.

Implications for conservation

The observation that sea turtle hybrids are usually found in very low frequencies worldwide, but can reach as much as 43% of nesting females from a short stretch of the Brazilian coastline seems to be, by itself, an important conservation issue. However, we still need to identify the primary causes and adaptive consequences of this regional phenomenon.

The considerable portion of hybrids in the Brazilian population might not be seriously threatening the conservation of the parental species at present, but further studies and special management measures should be taken in the Brazilian population, because it is the only known region in the world with such high rates of hybridization. It is essential to identify the causes of this hybridization event and to characterize this hybrid swarm in terms of reproductive and survivorship parameters to establish if the process could result in an eventual decline of the sea turtle populations. In addition, a long-term genetic monitoring of this rookery is also advisable to assess if hybrid proportions are rising in the population.

Although the mtDNA is the most used marker for genetic analysis in turtles (Bowen & Karl 2007; Lee

2008), the discovery of hybrids in feeding aggregations and nesting sites with typical morphology of one parental species (diverse from some few hybrids with noticeable mixed morphology found in nesting sites) indicates that the typing of only mtDNA markers may not be enough to delineate conservation strategies in areas where hybrids are common. Comparing the performance of microsatellites with nuclear sequences, we can note that microsatellites are less informative for detecting hybridization between species. For this reason, we suggest that nuclear sequence variation (SNPs or haplotypes) should be always typed in addition to mtDNA.

Previous studies dealing with hybridization between sea turtle species suggested that transplantation of individuals and other human interferences may be the cause of several cases of hybridization like between *L. kempii* and *C. caretta* (Karl *et al.* 1995; Stuart & Parham 2007). Hitherto, no indication has been found that transplantation of eggs or any managing strategies are causing the high frequency of hybrids in Brazil, as suggested by Karl *et al.* (1995) for *Lepidochelys kempii* in Texas, USA. It is believed that the transplantation of 18 000 eggs from Mexico to Texas (over 2000 km) could have caused the modification of nesting behaviour and stimulated hybridization between *L. kempii* and *C. caretta* in the beginning of 1990s. The managing programme in Bahia State in Brazil started in the 1980s, and egg transplantation is currently only carried out when nests are located in urban zones with high rates of predation and erosion, or in beaches where monitoring is complicated. Even when transference is necessary, the hatcheries are never moved more than a few kilometres away from the original nest. More than 70% of all nests are maintained *in situ*, without any manipulation of the eggs. Given the high rates of hybridization and the occurrence of introgression in the population that nests in Bahia, we believe that hybridization started before the beginning of the management programme of turtles in Brazil, because it spans at least two generations of sea turtle hybrids as shown in the introgression evidence.

Several studies estimated the age-at-maturity (or age for the first reproduction) for marine turtles to vary from 40 to 60 years for *C. mydas* (Meylan & Donnelly 1999), 20–40 years for *E. imbricata* (Meylan & Donnelly 1999) and 22–29 years for *C. caretta* (Heppel 1998; Casale *et al.* 2011). Thus, to generate an introgressed individual will take at least 40 years, which is a minimal date for the beginning of the hybridization in Brazil. Our results indicate that sea turtle hybridization occurring in the Brazilian coast may be linked to overhunting and local warming of beaches due to coastal deforestation (Matsuzawa *et al.* 2002). These could be the direct causes of the recent decline of sea turtle populations, reaching its climax in the 1970s in Brazil, which could have triggered an increase in interspecies hybridization.

The singular evolutionary process that is happening with the marine turtles nesting in Brazil requires special monitoring of the population. Theoretically, this introgressive hybridization process may threaten the long-term parental species identity and is perhaps already affecting population fitness. A recent study (Marcovaldi *et al.* 2012) identified different migration patterns for *E. imbricata* × *C. caretta* hybrids through satellite-tracking of 'pure' and hybrid females. Within the hybrid females, they tracked three individuals that migrated to foraging sites of *C. caretta* in north Brazil, and a single individual that migrated to foraging grounds along the east coast where 'pure' *E. imbricata* individuals feed. This is clear evidence that hybrids derived from the same crossing (*E. imbricata* × *C. caretta*) may display distinct behaviour and probably also different feeding abilities. If deleterious consequences of this singular hybrid swarm are confirmed in the near future, management strategies should be envisaged to reduce the impact of this event and guarantee the integrity of these threatened parental species. Further studies involving nuclear markers, analysis of nesting viability, and behavioural ecology of these hybrids are also needed to better understand this evolutionary process.

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F.R.S S.T.V P.L-R S.M.V L.S.S designed the research, S.T.V S.M.V P.L-R E.M performed the laboratory research, L.S.S E.C.R G.L-J contributed with samples, S.T.V analysed data, F.R.S coordinated the study, S.T.V F.R.S wrote the paper. All authors reviewed the text and analyses and approved the final manuscript.

Data accessibility

All nuclear sequences were deposited in GenBank under Accession nos JF415092-JF415134. Sequence alignments and individual-by-individual sequence data information are available under DRYAD entry doi:10.5061/dryad.j5240. For complete individual-by-individual data, including sampling location, morphological type and GenBank Accession nos, please see Table S3.

Supporting information

Fig. S1 Networks of the five nuclear genes sequenced in this study.

Fig. S2 Estimation of the number of groups (K) using the program STRUCTURE.

Fig. S3 Factorial Correspondence Analysis on the first three axes produced with Genetix using microsatellite and sequence data for individuals of 'pure' species and different hybrid classes.

Fig. S4 Diagrams illustrating the different interspecific crossings investigated in this study: (A) *C. caretta* × *E. imbricata*, (B) *C. caretta* × *L. olivacea*, (C) *E. imbricata* × *L. olivacea*, and (D) *C. caretta* × *E. imbricata* × *C. mydas*.

Fig. S5 Output chart from the introgression analysis (admixture) in the program STRUCTURE using four microsatellite loci.

Table S1 Primers used to generate autosomal sequence data that are described in detail in the Methods section and Appendix S1.

Table S2 Description of samples used on the complete and filtered data set.

Table S3 Detailed genotype of the filtered data set generated in this study.

Appendix S1 Detailed protocols for genotyping RFLPs, autosomal sequences, and microsatellites.