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Research paper

Unravelling the hybridisation patterns of Abrolhos' sea turtles

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Abstract

Unravelling the hybridisation patterns of Abrolhos' sea turtles. Hawksbill and loggerhead sea turtles form interspecific hybrids on the Brazilian beaches much more frequently than in any other part of the world. We have characterised the turtle population of the Abrolhos Archipelago (Brazil), by comparing the genetic profiles of hatchlings to assess their introgression level and the reproductive preference of parental females. We genotyped four species-specific nuclear markers in sea turtle hatchlings, including multiple siblings from the same nests. Our data revealed multiple paternity mates and backcrosses between hybrids and both parental species. We also show compelling evidence supporting the hypothesis that male hybrids can generate descendants. It also highlights the unpredictable mating behaviour of sea turtle hybrids, which can reproduce with either hybrids or parental species.

Key words: Hawksbills, Loggerheads, Multiple paternity, Conservation genetics, Introgression

Introduction

Interspecific hybridisation is a relatively common phenomenon in vertebrate taxa, even though it is usually found in low frequency and in particular contact zones (Allendorf et al 2001). However, hybridisation might impose a big challenge to conservation since it may lower population fitness (Arriaga-Mayorga et al 2024). Although interspecific hybrids can be naturally found in many taxa, and even be eventually associated with the formation of new species, particularly in plants (Butlin and Smadja 2018), it is commonly caused or augmented by direct and indirect human impacts (Harbicht et al 2014).

The interspecific hybridisation associated with anthropogenic causes can be a serious conservation concern. Human-mediated drivers such as animal translocation and population decline may increase the likelihood of interspecific crosses (Arriaga-Mayorga et al 2024). Translocation can lead to the encounter of species that would not naturally coexist spatially, while population decline reduces the number of conspecific mates available, potentially increasing the changes of individuals to breed with closely related species (Pinto et al 2016, Howe et al 2018). Interspecific mating is especially favoured in regions with low population densities and biased sex ratios, which may result in hybrid offspring that commonly show a reduction of survival or fertility, though they can sometimes be at least partially fertile (Mallet 2005). The fitness decline of interspecific hybrid populations is known as outbreeding depression and is considered an important issue for biodiversity conservation (Allendorf et al 2001, Arriaga-Mayorga et al 2024).

It has been proposed that between 1-10% of animals hybridise with at least one species, and the viability of hybrids is usually related to the divergence time of the parental species, i.e., recently divergent taxa commonly have a higher probability of forming viable hybrids (Mallet 2008). However, sea turtles (Cheloniidae) are intriguing

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exceptions to this common pattern (Vilaça et al 2022). The Cheloniidae family encompasses six sea turtle species that include *Caretta caretta* (loggerhead turtle, *Cc*), *Eretmochelys imbricata* (hawksbill turtle, *Ei*), *Chelonia mydas* (green turtle, *Cm*), *Natator depressus* (flatback turtle, *Nd*), *Lepidochelys kempii* (Kemp´s ridley turtle, *Lk*) and *Lepidochelys olivacea* (olive ridley turtle, *Lo*), which display a general pattern of speciation with gene flow (Vilaça et al 2021). Sea turtle species of the Cheloniidae family frequently hybridise even though the family diverged around 43 million years ago (Nash and Ryan 2023).

The loggerhead (*Cc*) and the hawksbill (*Ei*) sea turtles are classified as vulnerable and critically endangered species, respectively, according to the Red List of the International Union for Conservation of Nature (IUCN) (Casale and Tucker 2017 for *Cc;* and Mortimer and Donnelly 2008 for *Ei*). Their threatened status is a direct result of human activities such as predatory and accidental fishing, consumption of eggs, meat and hooves, marine contamination and the destruction of coastal habitats (Wallace et al 2011). In addition to the direct human causes associated with the decline of sea turtle populations described above, there are also indirect causes for which humans are primarily responsible. For example, climate change (IPCC 2021) poses the greatest threat in the current scenario as fluctuations in sea-level should destroy many nesting sites, and rising temperatures lead to an increase in the births of females, as sex determination in turtles is based on the temperature at which the eggs are hatched (Martins et al 2022).With the constant and rapid rise in global average temperatures, the sex ratio of turtles is expected to change in many places, with more females being produced than males due to temperature-dependent sex determination. This feminization phenomenon has already been documented in sea turtle populations, including on nesting beaches in the Brazilian states of Bahia and Sergipe. In these locations, the decline of coastal vegetation has reduced shaded nesting areas, leading to increased sand temperatures during incubation and consequently contributing to female-biased sex ratios (Marcovaldi et al 1997). Such shifts in sex ratio can reduce the availability of males in the population, potentially affecting reproductive dynamics and mating opportunities.

Another intriguing phenomenon affecting conservation of sea turtles is the hybridisation between some species observed frequently in nesting colonies in North-eastern Brazil. While hybridisation in sea turtles is typically found in low frequencies worldwide (Lara-Ruiz et al 2006), the hybrid nesting females in the North-eastern Brazilian coastline can exceed a frequency of 25 %, reaching up to 40 % in some nesting sites (Lara-Ruiz et al 2006, Reis et al 2009, Brito et al 2020, Vilaça et al 2021, Almeida et al 2023). This high rate is facilitated by the overlap in nesting areas and breeding times among *Ei*, *Cc*, and *Lo* in north-eastern Brazilian nesting sites (Bahia, Sergipe and Alagoas states), coupled with declining populations and skewed sex ratios (Vilaça et al 2012, Soares et al 2017, Chaloupka and Marcovaldi 2007). Despite the strong philopatric behaviour of sea turtles, which typically leads females (and likely males too) to return to their natal beaches for nesting, changes in habitat availability can disrupt these patterns and increase hybridisation opportunities. Thus, together with the decrease in populations with biased sex ratios, the overlapping of nesting sites facilitates the encounter of different species and their hybridisation (Vilaça et al 2012, Soares et al 2017).

In this work, we focused on analyses of hatchlings from Abrolhos Marine National Park (Abrolhos), an archipelago of five tiny islands 70 km off the coastline of the Bahia state in Brazil. The hatchlings include the offspring of first generation (F1) hybrids that show signs of introgression from both parental species as suggested by genomic analyses (Arantes et al 2020a). We compared data from purebred sea turtles (*Cc*, *Ei*) and *Cc* x *Ei* F1 hybrids (descended from *Cc* and *Ei* crosses) from Abrolhos to expand knowledge of hybridisation dynamics and determine genealogical relationships between hatchlings and parents. In addition, F2 offspring of first-generation hybrids (*Cc* x *Ei* F1) in Abrolhos have shown backcrossing (introgression) with both parental species as determined by multilocus sequencing and triple RAD analyses (Arantes et al 2020a). We used four species-specific haplotypes sequenced using a PCR approach to increase genetic information from poor quality samples (decayed tissues from dead animals), and identified hybrid patterns of mating and the genetic composition of hatchlings.

The existence of sea turtle nesting sites in Abrolhos is relatively new and occurs only in small numbers (Arantes et al 2020a). Therefore, it is important to understand the ecology and evolution of these animals, focusing on the likely origin of nesting females (and their copulating males) and the impact on the fitness of the local population. Our findings provide new insights into hybrid and non-hybrid female mate choice in sea turtles, highlighting multiple paternity among different species and hybrids within nests, and supporting informed local conservation management.

Material and methods

Sampling and DNA extraction

The study was carried out by using DNA samples from previously collected tissues of dead hatched hatchlings (Arantes et al 2020a) from the Abrolhos Archipelago (17° 20' - 18° 10'S and 38° 35' - 39° 20'W). Altogether, 152 hatchlings from 21 different nests were analysed. The individuals were found in the nests in three different states based on their developmental outcomes: successful hatchlings that emerged from the egg and exited the nest to reach the ocean (if not predated beforehand), counted based on empty eggshells; dead hatchlings that were fully developed and successfully broke free from their eggshells but died before exiting the nest; and unhatched hatchlings that remained inside their eggs and failed to hatch while their siblings had already emerged, likely due to developmental malformations that prevented them from completing development and breaking through the eggshell.

Nests were identified as distinct clutches based on their physical separation on the beach, with each nest representing an individual egg-laying event. We assumed one mother per nest, as each nest represents a single clutch laid by one female (Miller 1997). We analysed multiple offspring per nest to identify full-sibling groups and reconstruct parental genotypes based on Mendelian inheritance patterns.

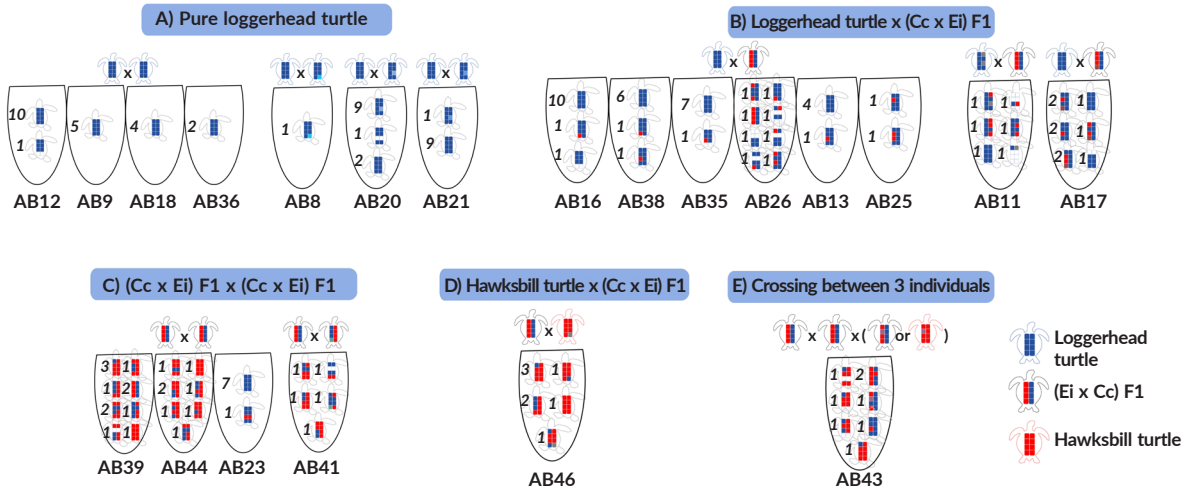

Fig. 1. Identification of individuals at each locus between *C. caretta*, *E. imbricata* or hybrid individuals separated by nest. The turtle silhouettes above each nest or cluster of nests illustrate the most likely parents of the sibling hatchlings. Alleles for the loci 3061, 64188, 76958 and 856 are shown from top to bottom. The most common allele for each species at each locus is represented by a consistent colour across all loci: red for *E. imbricata* (*Ei*) and blue for *C. caretta* (*Cc*). Less common or rare alleles from the same species are shown in shades of the same colour family: warm tones (shades of red/orange) for *Ei*-associated alleles and cold tones (shades of blue/cyan) for *Cc*-associated alleles. Gray alleles are non-diagnostic alleles (equally distant from both species) and white blocks represent missing data. The numbers in front of each hatchling genotype identify the number of individuals with the proposed genotype per nest. A, Crossings between two pure *C. caretta*; B, Crossings between F1 hybrids and pure *C. caretta*; C, Crossing between male and female hybrids (F1); D, Crossings between F1 hybrids and pure *E. imbricata*; and E, Crossings involving > 2 males, most likely between an F1 hybrid female and an F1 hybrid male, and another F1 hybrid or a pure *E. imbricata*.

The putative hatchling species assignment as pure loggerhead (*Cc*), pure hawksbill (*Ei*), or hybrid (*Cc* x *Ei*) was done based on the individual morphology (although the morphological species assignment of hatchlings is dubious), and few others were nestlings from a mother with a known morphological and molecular identification (Arantes et al 2020a). All samples were from stillborn individuals, whether hatched or not (Arantes et al 2020a).

Genetic analysis of the 21 nests revealed distinct reproductive patterns and nest structures (fig. 1). Seven nests showed pure loggerhead turtle ancestry (both parents *Cc*), eight nests resulted from crosses between F1 hybrids and pure loggerheads (*Cc* x *Ei* F1 × *Cc*), three nests originated from matings between two F1 hybrids ((*Cc* x *Ei*) F1 × (*Cc* x *Ei*) F1), two nests came from crosses between F1 hybrids and pure hawksbills ((*Cc* x *Ei*) F1 × *Ei*), and one nest indicated multiple paternity possibly involving three individuals. This nest structure demonstrates diverse mating strategies occurring in the Abrolhos Archipelago, including intraspecific breeding, interspecific hybridization, and backcrossing events between hybrids and parental species.

Samples were individually named by a code composed of AB (Abrolhos) + Nest Number + F + Hatchling number, such as AB39F9, which means hatchling 9 from nest 39 of the Abrolhos Archipelago. Dead hatchlings were originally collected between September and March, from 2017 to 2019 by the Marine National Park of Abrolhos team, and deposited at the Taxonomic Collections Centre (CCT) at the Federal University of Minas Gerais (UFMG) in ethanol 70%. Genetic resources access is reported at SisGen-Brazil under registration number A03A2C2.

DNA extraction of tissue samples was performed using a modified phenol-chloroform protocol (Sambrook and Russell 2001). The extracted DNA samples were quantified and normalized to a 30 ng/µL concentration.

Anonymous nuclear markers amplification and sequencing

Four species-specific anonymous markers, namely 856, 3061, 64188, and 76958 were identified and developed in a previous genomic survey with genomic ddRAD sequencing (Arantes et al 2020b). These anonymous PCR-based markers were ancestry-informative loci selected to work as species identifiers, called species-specific markers, that were successfully used to identify hybrids, including some hatchlings and nesting females (Arantes et al 2020a, 2020b). These loci were analysed as haplotypes composed of two to four diagnostic sites (SNPs) found to discriminate mainly between the two selected species, *Cc* and *Ei* (table S1). They were used in the analysis of several individuals from the Brazilian coast, including *C. caretta*, *E. imbricata*, and hybrids.

For the PCR amplification of the loci, the previously designed primers for the four selected loci were used (Arantes et al 2020a, 2020b) (table S1). The PCR was performed with a final volume of 15 µL, following the amounts: 200 µM of dNTP, 0.5 units of Platinum™ Taq DNA polymerase (Invitrogen™ by Life Technologies™), 1.5 mM (up to 4 mM, depending on sample quality) $MgCl_2$, 0.5 µM of forward and reverse primers, 0.5 mg/mL of Bovine Serum Albumin (BSA) and ~60 ng of genomic DNA in a 1X reaction buffer (Invitrogen™ by Life Technologies™).

PCR cycling was performed with an initial denaturation cycle of 95°C for five minutes, 35 denaturation cycles of 95°C for 30 seconds, and variable annealing temperatures for 40 seconds (59°C, 56°C, 58°C and 64°C for 856, 3061, 64188, and 76958, respectively), extension to 72°C for one minute, and a final extension at 72°C for seven minutes (Arantes et al 2020b). The effectiveness of PCR was verified by electrophoresis in a 1% agarose gel and the amplified PCR products were purified by precipitation using a polyethylene glycol (PEG) solution with a concentration of 20 mM.

Sequencing was performed using the BigDye Terminator Cycle Sequencing kit (Thermo Fisher Scientific) according to the standard protocol. Amplicons were sequenced on the ABI 3130xl DNA sequencer (Thermo Fisher Scientific).

Generation of phased nuclear sequences

The SeqScape v2.6 software (Thermo Fisher Scientific) was initially used to verify the quality of the sequenced forward and reverse reads for all four markers/loci. To reconstruct the haplotypes of each parental chromosome, phasing was performed using DNAsp v5 (Librado and Rozas 2009) from unphased data (with ambiguous genotypes) by using the algorithms provided by PHASE (Stephens et al 2001, Stephens and Donnelly 2003), fastPHASE (Stephens and Scheet 2005) and HAPAR (Wang and Xu 2003). Haplotype phasing is essential for determining which combination of alleles occur together on each chromosome copy. For individuals heterozygous at multiple loci, phasing algorithms reconstruct the most likely haplotype configurations based on population-level linkage disequilibrium patterns and Hardy-Weinberg expectations. All individuals with phased haplotypes were added and aligned using the DNAsp v5 program, together with all *Cc* and *Ei* haplotypes previously characterized for these loci (GenBank accession numbers are available in table S2). This alignment generated a species-specific haplotype distribution. Haplotypes presenting alleles that differed from the expected parental patterns were analysed in greater detail (see Discussion).

Network and phylogenetic analyses

The phased haplotypes obtained for each locus were submitted to phylogenetic analysis in the program Network (Fluxus Technology 2021) to generate a haplotype network. In this phylogenetic network, the molecular genealogy of all haplotypes connected by mutation steps was analysed, as well as the population frequency of each haplotype (fig. 2). Species-specific haplotypes for five sea turtle species that spawn in Brazil, including *Cc* and *Ei*, were identified previously by Arantes et al (2020b), including all nuclear haplotypes to be used as controls for pure *E. imbricata* and *C. caretta* turtles. We inferred that both parents must be hybrids when offspring exhibited specific multilocus patterns. Specifically, if an offspring is homozygous for *Cc* alleles at one or more loci and simultaneously homozygous for *Ei* alleles at one or more different loci, this pattern can only arise if both parents carried alleles from both species. For example, if an offspring is CcCc at locus A and EiEi at locus B, then both parents must have contributed a *Cc* allele at locus A (both parents carry *Cc*) and both parents must have contributed an *Ei* allele at locus B (both parents carry *Ei*). This multilocus homozygosity for different species at different loci is the diagnostic signature of hybrid × hybrid crosses and cannot result from hybrid × purebred matings.

The phased haplotypes from Abrolhos individuals and purebred controls (known species) were also submitted to an analysis for all loci in SliptsTree (Hudson and Bryant, 2006) using the Neighbor Net package (Bryant and Moulton 2004) with genetic distance matrices (Neighbor-Joining, Saitou and Nei 1987) between all samples and loci combined (Genpofad distances). This analysis enables the joint study of multiple haplotypes of individuals from different populations, considering all the sequenced regions (four markers). Genpofad distances were calculated in default mode to compile distance matrices for all loci studied and to create the SliptsTree input, using the pofadinr R package (Joly et al 2015).

F1 individuals were inferred when phased haplotypes showed only *E. imbricata* alleles on one chromosome and only *C. caretta* alleles on the other chromosome. This pattern indicates that F1 hybrids inherit 50% of their genetic material from each parental species without recombination. Hybrid parentage was inferred when an individual showed at least one locus homozygous for *C. caretta* alleles and another locus homozygous for *E. imbricata* alleles. Because each parent contributes one allele per locus, this pattern is only possible when both parents already carry alleles from both species (*Cc*×*Ei*), allowing them to produce offspring that are homozygous for *Cc* at some loci and homozygous for *Ei* at others.

Results

Ascertainment of the species-specific haplotype origin

To confirm that our four nuclear loci (3061, 64188, 76958, 856) are truly species-diagnostic, we compared phased haplotypes from Abrolhos hatchlings against reference haplotypes from pure individuals of five sea turtle species collected across Brazilian nesting sites (Arantes et al 2020a, 2020b; tables S2 and S3). Phylogenetic network analyses revealed complete haplotype differentiation between *C. caretta* and *E. imbricata* across all four loci, with no allelic overlap observed (fig. S1 and S2). The markers successfully discriminated not only between *Cc* and *Ei*, but also among all five species analysed (n = 22 reference specimens; fig. S1), confirming their species-specificity and diagnostic power.

Within pure individuals, we observed intraspecific polymorphism consistent with expected population-level variation. For example, pure E. imbricata specimens R0592 and R0176 were heterozygous at locus 856 (fig. S2C), carrying two different *Ei*-specific alleles (Ei4/Ei5). Critically, however, no pure individual carried alleles from multiple species at any locus. This complete species-level sorting validates the reliability of our markers for hybrid detection: individuals carrying both *Cc* and *Ei* alleles must have hybrid ancestry. These results provide a robust foundation for inferring hybrid status and parental genotypes from offspring multilocus genotypes.

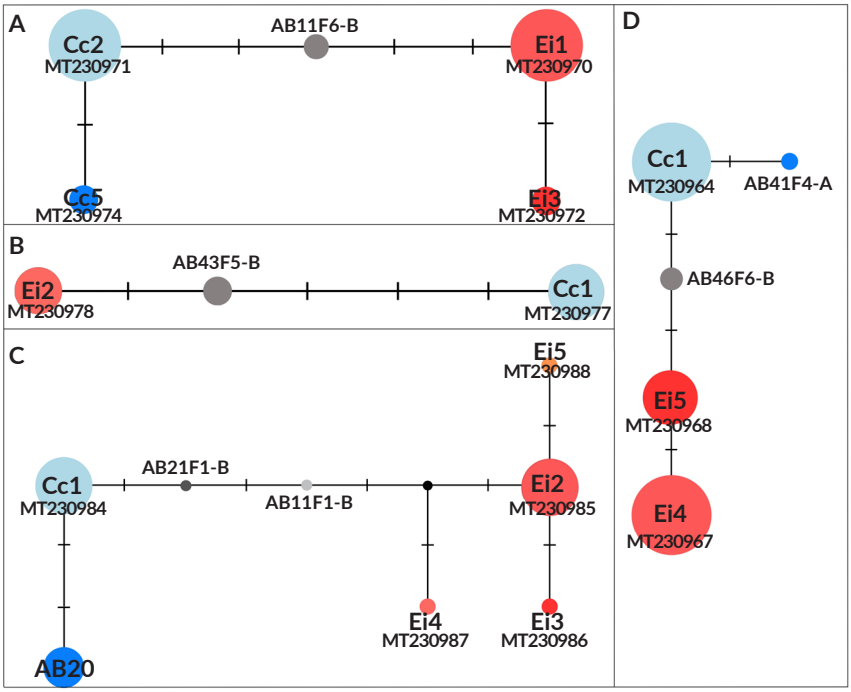

Fig. 2. Haplotype networks for four loci in individuals from Abrolhos. The size of each circle is proportional to the number of individuals carrying that haplotype. Blue circles represent *C. caretta* (*Cc*) haplotypes, red circles represent *E. imbricata* (*Ei*) haplotypes, and grey circles represent intermediate haplotypes. Each dash represents a single mutational step. 'Mv' represents a median vector (a hypothetical, unsampled, or ancestral haplotype inferred by the program to connect observed haplotypes). A, Locus 3061. Haplotypes Ei1, Cc2, Ei3, and Cc5 have been previously described. A new intermediate haplotype was found in individual AB11F6, positioned between Cc2 and Ei1; B, Locus 64188. Haplotypes Cc1 and Ei2 have been previously described. A new intermediate haplotype was found in individual AB43F5, closer to *Ei* than to *Cc*; C, Locus 76958. Three new haplotypes were identified: in individual AB11F1, in individual AB21F1, and in nine individuals from nest AB20. Cc1 and Ei2 were the most common haplotypes; D, Locus 856. New haplotypes were found in individuals AB46F6, AB8, and AB41F4. The most frequent haplotypes were Cc1 and Ei4.

Haplotype diversity in Abrolhos population

Having validated marker specificity, we characterized genetic diversity within the Abrolhos population by constructing haplotype networks including all 152 sequenced hatchlings (fig. 2). We expected pure parental species to exhibit only species-specific alleles (*Cc* individuals: all blue haplotypes; *Ei* individuals: all red haplotypes), while F1 hybrids should be heterozygous at each locus (one blue, one red allele per locus; fig. 2). Backcrossed (F2) offspring would show variable combinations depending on parental contributions.

Our analysis identified all 16 previously described haplotypes (table S2) and revealed several novel variants. At locus 76958, we discovered four additional species-diagnostic SNPs in a new haplotype (CcxEi9_76958) found in the confirmed hybrid individual AB46F5 (table S4). Across these four sites, all *C. caretta* individuals display the A-A-C-C haplotype pattern, while all *E. imbricata* consistently show T-T-A-T, and AB46F5 exhibits the fully heterozygous W-W-M-Y genotype. Although this marker shows strong diagnostic potential for future studies, we excluded it from downstream analyses due to incomplete sequence coverage across individuals.

Additional novel haplotypes were identified across all four loci (fig. 2; table S4). At locus 3061, individual AB11F6 carried a previously undescribed allele (Cc8_3061) positioned equidistant from both Cc2 and Ei1, preventing definitive species assignment. At locus 64188, we identified two novel haplotypes: Cc8_64188 in AB38F8 (clustering within Cc1) and CcxEi9_64188 in AB43F5 (intermediate between Cc1 and Ei2). Locus 76958 yielded three additional novel haplotypes (CcxEi6_76958, Cc8_76958, Cc7_76958) in individuals AB11F1, AB21F1, and nine individuals from nest AB20. Finally, at locus 856, we detected two undescribed haplotypes: Cc9_856 shared by AB8 and AB46F6, and Cc8_856 exclusive to AB41F4.

The discovery of these novel haplotypes, some intermediate between parental species and others representing new intraspecific variants, reveals greater genetic diversity in the Abrolhos population than previously recognized. Intermediate haplotypes may represent recombinant alleles formed through hybridization, retention of ancestral polymorphisms, or incomplete lineage sorting, hypotheses which will be addressed in the Discussion.

Parental genotype reconstruction and mating patterns

Using multilocus genotypes from multiple siblings per nest, we reconstructed the most parsimonious parental crossing schemes for all 21 nests (fig. 1). This approach

leverages Mendelian inheritance patterns: the combination of alleles present across siblings within a nest constrains the possible parental genotypes that could have produced them.

Seven nests (AB8, AB9, AB12, AB18, AB20, AB21, AB36) contained offspring carrying only *C. caretta* alleles across all four loci, consistent with pure *Cc* × *Cc* crosses. Nine nests (AB11, AB13, AB16, AB17, AB23, AB25, AB26, AB35, AB38) exhibited offspring with mixed *Cc* and *Ei* alleles in patterns indicating one purebred *Cc* parent and one F1 hybrid parent (*Cc* × F1 backcrosses; fig. 1B). One nest (AB46) showed evidence of a purebred *Ei* × F1 hybrid cross (fig. 1D).

Critically, five nests (AB23, AB39, AB41, AB43, AB44) contained offspring whose genotypes could only be explained by crosses between two F1 hybrid parents (fig. 1C). For instance, individual AB44F1 is homozygous for *Ei* alleles at loci 3061, 64188, and 856, yet homozygous for *Cc* alleles at locus 76958. This pattern —homozygosity for different parental species at different loci— can only arise if both parents carried both *Cc* and *Ei* alleles, indicating that both parents were hybrids. Similar patterns were observed in individuals AB39F6, AB39F7, AB41F1, AB41F2, AB44F5, and others (green squares in fig. 3), providing genetic evidence of F1 × F1 crosses producing F2 offspring. In this analysis is possible to identify introgression of both species in the F2 offspring, depending whether the nest (or individuals) are closer to the pure individuals (in both extremes of fig. 3).

Of the 21 nests analysed, 11 nests (52 %) showed patterns consistent with purebred *C. caretta* parentage (fig. 1A), 7 nests (33 %) showed evidence of Cc×Ei crosses (one purebred parent and one F1 hybrid parent; fig. 1B), and 3 nests (14 %) indicated Cc×Ei × Cc×Ei crosses (both parents being hybrids; fig. 1C). Overall, 10 of 21 nests (48 %) contained hybrid offspring, indicating that hybridization is common in this population.

Evidence for fertile hybrid males

The F1 × F1 hybrid crosses documented above provide the first genetic evidence that hybrid males are fertile and reproducing in this population. Previous studies confirmed F1 hybrid females through direct sampling during nesting (Arantes et al 2020a), but male reproductive contributions remained theoretical because males never come ashore and cannot be directly sampled (Vilaça et al 2012, Soares et al 2018).

The presence of F2 offspring with genetic signatures requiring hybrid male parentage (e.g., AB44F1, AB39F6, AB41F1) demonstrates that: (1) hybrid males reach sexual maturity, (2) hybrid males successfully mate and fertilize eggs, and (3) hybrid male contributions produce viable offspring capable of at least completing embryonic development. Since each nest represents a single female's clutch, and we observe F1 × F1 crossing patterns in five nests, at minimum five different hybrid males contributed to reproduction in our sample.

This finding fundamentally changes our understanding of gene flow in this hybrid zone from unidirectional (hybrid females × purebred males) to bidirectional (both hybrid sexes contributing to introgression).

Multiple paternity

Although our markers have lower resolution than microsatellites for paternity analysis, examining multiple siblings per nest enables detection of multiple paternity when offspring genotypes cannot be explained by a single paternal contribution. Nest AB43 provides evidence of multiple paternity, with offspring showing genetic compositions consistent with contributions from more than one male (fig. 1E). Specifically, offspring in this nest exhibit combinations of 'pure' *Ei*, *Cc* × *Ei* hybrid, and novel rare haplotypes that suggest the nesting female mated with at least two males —possibly including both an F1 hybrid and either another F1 hybrid or a pure *Ei* male carrying a rare allele at locus 64188.

This represents the first documentation of multiple paternity in Abrolhos nesting turtles, a phenomenon known to occur in 35.7 % of nests on other Brazilian beaches (Soares et al 2018) but not previously confirmed in this population. The biological plausibility is well-established, as female sea turtles can store viable sperm from multiple males for extended periods (de Borba et al 2019).

Multilocus networks

To visualize relationships among all individuals simultaneously across all four loci, we constructed a Neighbor-Net phylogenetic network using concatenated genetic distance matrices (fig. 3). Pure *C. caretta* (CC1 and CC2 in fig. 3) and *E. imbricata* (R0028 and R0206 in fig. 3) individuals cluster at opposite extremes of the network, with F1 hybrids positioned intermediately (shown as spheres). F2 and backcrossed individuals (squares) exhibit varying degrees of admixture, forming a genetic continuum between parental species (fig. 3).

Several individuals initially suspected to be hybrids based on morphology or single-locus data clustered with purebred references in the multilocus network. For example, AB39F12, AB44F6, and AB46F7 clustered with pure *E. imbricata* (near R0028), while AB11F5 grouped with pure *C. caretta* individuals (CC1, CC2, CC5) and the AB9 nest offspring. These cases likely represent F2 individuals that inherited predominantly one parental species' alleles at our four loci due to Mendelian sampling (12.5 % probability with four unlinked markers), illustrating the limitations of marker number and the value of the multilocus network approach for visualizing overall genetic similarity.

Discussion

This approach based on PCR and Sanger sequencing was very successful to obtain ancestry-informative genotypes for many individuals that were precluded by the genomic approaches due to the poor-quality of available DNA samples coming from dead (and decaying) hatchlings.

In a previous study, it was demonstrated that hybrid female turtles were reproducing in Abrolhos and generating viable offspring (Arantes et al 2020a). However, the existence of fertile hybrid males generating offspring was only a theoretical hypothesis (Vilaça et al 2012) until now. Therefore, this study provides compelling evidence for the existence of fertile hybrid males and

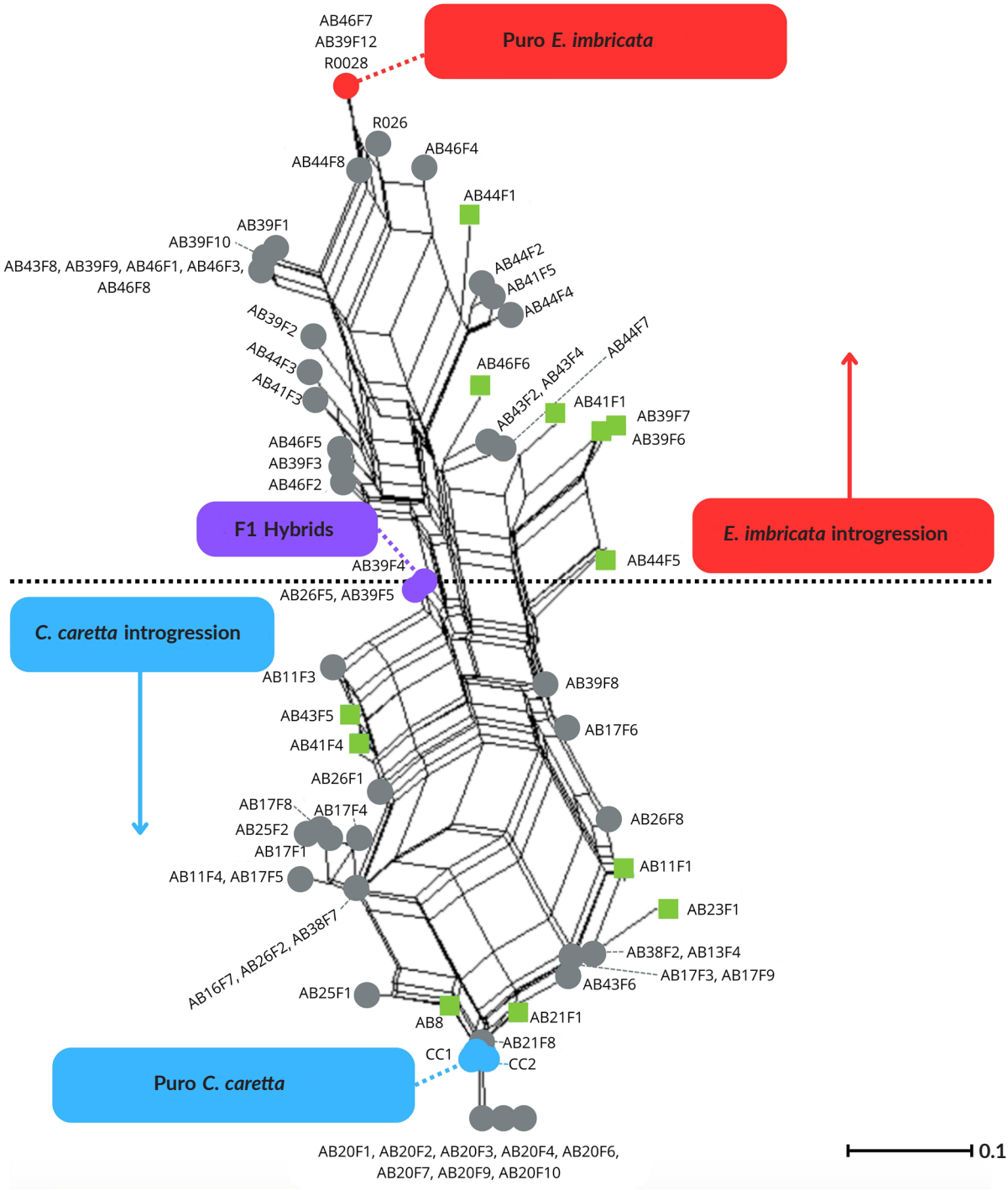

Fig. 3. Neighbor-Net analysis of Abrolhos individuals based on a concatenated genetic distance matrix of four markers (3061, 64188, 76958, and 856). The network illustrates genetic relationships and introgression patterns among sampled individuals. Coloured circles represent pure parental species, with red indicating *Eretmochelys imbricata* and blue indicating *Caretta caretta*. Purple circles denote F1 hybrids with intermediate genetic composition, positioned centrally between the two parental clusters. Gray squares represent introgressed individuals (F2 or later generation hybrids resulting from backcrossing between F1 hybrids and parental species), while green squares identify introgressed individuals with both hybrid parents. Pure *E. imbricata* individuals cluster in the upper region (red oval), while pure *C. caretta* individuals cluster in the lower region (blue oval). F1 hybrids (purple circles) occupy an intermediate position, serving as a genetic bridge between the parental species. The dotted horizontal line demarcates the introgression boundary: individuals positioned above this line show *E. imbricata* introgression (backcrossed primarily into *E. imbricata*), while individuals below show *C. caretta* introgression (backcrossed primarily into *C. caretta*). The reticulated structure reflects genetic admixture and complex evolutionary relationships resulting from bidirectional gene flow between these species. Branch lengths are proportional to genetic distances, and individual specimen IDs are labelled throughout the network.

possible multiple paternity involving loggerhead and hawksbill parental turtles in the Abrolhos Archipelago of North-eastern Brazil. Although multiple paternity is a common phenomenon in marine turtles (Erickson et al 2015), this is the first study demonstrating this reality in Abrolhos nesting turtles —and with hybrid males.

The hybrid origin of these hatchlings was inferred based on previous evidence of F1 hybrid females nesting in Abrolhos and mating with both parental species (*C. caretta* and *E. imbricata*), as confirmed by a reduced-representation genome analysis (Arantes et al 2020a). Given this scenario, multiple crossing events are possible, and both F1 and backcrossed hatchlings are expected. While the presence of hybrid females has been confirmed through genetic analysis of nesting adults, sampling hybrid males is more challenging since they do not come ashore. Our analysis based on multiple sibling hatchlings enables us to recognize that hybrid males may also be mating in Abrolhos. It is important to state that there was a large variation in the sampling number of dead hatchlings per nest (assumed to belong to a single mother), from 1 to 12 individuals, with an average 7.2 of per nest (fig. 1).

The discovery of fertile hybrid males represents a paradigm shift in our understanding of sea turtle reproductive biology and hybrid zone dynamics. Previously, hybridization research in sea turtles focused primarily on female hybrids, as they could be directly sampled and genotyped during nesting activities (Arantes et al 2020a, Vilaça et al 2021). Male reproductive contributions remained largely theoretical (Vilaça et al 2012, Soares et al 2018). Our genetic evidence of hybrid-hybrid pairings and the identification of F2 offspring with genetic signatures requiring hybrid male parentage demonstrate that males are actively contributing to introgression. This has critical implications for understanding gene flow directionality in this hybrid zone: rather than unidirectional introgression through hybrid females mating with purebred males, bidirectional gene flow is occurring through both hybrid females and hybrid males. This dynamic substantially increases the potential for genetic admixture and may accelerate the rate of introgression in the Abrolhos population. Furthermore, the existence of reproductively active hybrid males suggests that F1 hybrids of both sexes can reach sexual maturity and successfully reproduce, challenging previous assumptions about hybrid fitness barriers in marine turtles. However, given the highly female-biased sex ratios documented in Brazilian sea turtle populations (Marcovaldi et al 1997, Godfrey et al 1999), with hawksbill and loggerhead hatchlings exceeding 90% and 82.5% female respectively, the proportional contribution of hybrid males to the breeding population may be constrained by their relative scarcity compared to females. Future research incorporating molecular sexing of hybrid hatchlings could reveal whether sex-biased mortality further affects the male-to-female ratio among hybrids specifically.

It is important to acknowledge a key limitation in our inference of male hybrid fertility: all genetic evidence comes from deceased hatchlings that did not survive to enter the ocean. However, previous research has demonstrated that hybrid offspring in this population are indeed viable. Arantes et al (2020a) analysed both living and dead hatchlings from the same Abrolhos population and found that hybrid nests, while showing reduced hatching success compared to pure *C. caretta* nests, still produced viable hatchlings capable of reaching the sea. Their study documented that hybrid females successfully reproduce and generate viable offspring when mating with parental species, establishing that F1 hybrids can produce viable F2 offspring. Our current findings extend this understanding by providing genetic evidence that hybrid males are also contributing to reproduction. The hatchlings analysed in our study died at various developmental stages: some failed to emerge from eggs, while others successfully hatched but perished before reaching the sea. Combined with the evidence from Arantes et al (2020a) of viable hybrid offspring successfully emerging and entering the ocean, our results demonstrate that hybrid males are probably capable of producing viable offspring, though definitive tracking of these individuals to reproductive maturity remains challenging due to the difficulty of sampling males at sea.

It is important to note that our sample consisted exclusively of dead hatchlings found on the beach, which may be biased toward less viable individuals. Therefore, the observed proportion of F2 individuals (which may have lower fitness) could be overrepresented compared to the actual breeding population.

Our findings indicate a higher proportion of F2 individuals compared to F1 hatchlings (fig. 1) in Abrolhos, corroborating results of Arantes et al (2020a). However, due to the small number of markers used in this study, some F1 turtles identified here might also be F2 individuals whose introgressed alleles were not sequenced (missing data). In this context, there are some nests where we found hatchlings with typical F1 genotypes and others with F2 genotypes (e.g., AB26, AB39). For nest AB26 (fig. 1B), this can be explained by a parent being an F1 hybrid with alleles transmitted irregularly (entirely plausible for only four markers) —some offspring inherited alleles from predominantly one species while others inherited a mixture of alleles. This might also be the case for nests AB11, AB13, AB16, AB17, AB23, AB35, and AB38, which contain both F2 and seemingly F1 individuals. However, for nest AB39, the most likely explanation is that both parents are hybrids, which is likely also the case for nests AB23, AB41, and AB44 (fig. 1C). The ambiguous classification of nest AB23, which contains offspring consistent with both Cc × (Cc × Ei) and (Cc × Ei) × (Cc × Ei) crosses, could potentially be explained by multiple paternity, where different offspring within the nest resulted from matings with different males (one purebred and one hybrid). While our proposed crossing schemes represent the most parsimonious explanations based on known F1 hybrid females nesting in the population, we acknowledge that more complex generational scenarios, including potential reproduction by F2 individuals that have reached maturity or crosses between hybrids of different generations, cannot be definitively excluded without direct parental genotyping.

The probability for nests showing both F1 and F2 hybrids depends on the chromosome copy inherited from each parent by the offspring. Since alleles in the progeny are donated by parents randomly, an F1 hybrid parent has the same probability (50%) of donating alleles from each parental species, for each locus. However, all the

alleles donated by the hybrid parent may be sometimes from the same parental species, which means that there are offspring that can mistakenly appear as hawksbill purebreds in nests where it is known that the mother is a hybrid, as mentioned above. Therefore, the possibility of transmitting one particular haplotype (in four loci) rather than another is:

$$2(1/2)^4 = 0.125$$

This means that 12.5 % of the zygotes/eggs/hatchlings will have the genotype combination of only one species (for the four loci) even though one of the parental individuals was a F1 hybrid, which possesses both *Cc* and *Ei* alleles. In our study, the probability of mistakenly identifying an individual as purebred when it is actually F2 is also 12.5 %. This is the case for individual AB11F5 in nest 11 and individuals AB17F2 and AB17F7 in nest AB17 (table S5).

The most likely crossing originating some hatchlings in nests AB39, AB44, AB23 and AB41 considers that both parents are hybrids, a possibility never indicated by previous genetic studies until now (Soares et al 2017, Arantes et al 2020a, Soares et al 2021). It is expected when an offspring has inherited *Cc* and *Ei* alleles from both hybrid parents. Some hatchlings indicate that both parents are hybrids more clearly, such as individuals AB39F6, AB39F7, AB41F1, AB41F2, AB44F1, and AB44F5 (fig. 1 and table S5), which appear as green squares in the Neighbor-Net (fig. 3). Based on the analysis of a set of individuals of the same nest, we are capable of reconstructing the possible parental genotypes. Therefore, it is possible to assume that individuals AB39F6, AB39F7, AB41F1, AB41F2, AB44F1, and AB44F5 are the offspring of both hybrid parents, supporting the hypothesis of hybrid male parents in Abrolhos. These findings provide the first genetic evidence of hybrid-hybrid pairings in sea turtles, a scenario previously unreported. This suggests that males are also contributing to the ongoing hybridisation process, which can influence the evolutionary trajectory of these populations (see Arantes et al 2020a).

The robustness of our results depends on the diagnostic power of the genetic markers analysed. To confirm that the previously described loci were indeed species-specific, network analyses of allele/haplotype discrimination were performed. As demonstrated, there were no purebred individuals whose alleles/haplotypes were shared between two different species (fig. 3).

To accurately assess hybridisation and introgression, our analysis included multiple siblings from the same nest. This is essential because relying on a limited number of genetic markers and samples can lead to misinterpretations, potentially missing signals of introgression. Clear examples of this occurred in nests AB13, AB16, AB23, AB35 and AB38. In previous research (Arantes et al 2020a), a single individual from each of these nests was analysed and they were classified as pure loggerhead nests. In the current research, using multiple siblings from the same nests, the alleles of *Cc* and *Ei* species were identified in the hatchlings, which demonstrates that they are actually F2 hybrids (fig. S5). Another notable example is nest AB17, in which the analysis of the two individuals that appeared to be 'pure-like' loggerheads would have led us to conclude that AB17 was a nest of a 'pure female' when it is not. The inspection of a larger number of hatchlings demonstrated that some are actually backcrossed hybrids (F2), and few were further confirmed by reduced representation genomic data (Arantes et al 2020a). This finding highlights the importance of analysing multiple hatchlings per nest and increasing the number of genetic markers to obtain a more comprehensive understanding of the parental contributions to the hatchlings' genetic composition, allowing us to identify hybridisation patterns and parental characteristics that were previously overlooked. Our species-specific PCR-based markers allowed to sequence many poor-quality samples derived from dead hatchlings, which were previously used unsuccessfully to generate RAD sequencing libraries (Arantes et al 2020a).

Alternative crossings to the ones proposed in fig. 1 might be considered, especially if we consider that multiple paternity is a common phenomenon in marine turtles (Erickson et al 2015), reaching 35.7 % of studied nests on Brazilian beaches (Soares et al 2018). However, we consider that the proposed crossing is the most parsimonious based on our knowledge about the population, known to have hybrid female individuals (Arantes et al 2020a). Nest AB43, for instance, shows evidence of more than one male contributing to the offspring's genomic composition (fig. 1E) and therefore it indicates the possibility of multiple paternity although rare in populations from the United States (Lasala et al 2019), further studies are needed to fully access the amount of multiple paternity in population worldwide and mainly in highly hybrid groups, such as the Abrolhos one. This pattern is biologically plausible given that sea turtles can store viable sperm from multiple males for extended periods (de Borba et al 2019), which can generate multilocus offspring signatures consistent with multiple paternity even when mating events are not directly observed. In AB43 nest, one of the parents is an F1 hybrid (*C. caretta* x *E. imbricata*) and the other parents are either *i*) *Cc* x *Ei*, or *ii*) pure *Ei* with a new allele in the 64188 locus. We assume that each nest has the genetic contribution of only one female since it carries the eggs and lay them in one specific spot (Wright et al 2012, Broderick at al 2016, Soares et al 2018). Therefore, the different genetic background seen in this nest should come from different males. The hatchlings from this nest have interesting genetic composition since only one presents a rare allele (AB43F5) and others have 'pure' *Ei* and *Cc* x *Ei* haplotypes, indicating that the father is either a F1 hybrid or a pure *Ei*, both carrying the rare haplotype. However, for it to be possible, there needs to exist fertile hybrid males in reproductive age, which was previously theorized by Soares et al (2018) but has yet not been observed.

Backcrossed hatchlings identified previously with the same markers used in this research showed that female F1 hybrids can reproduce with males from both parental species, which demonstrated that the process of introgression might happen in either direction (Arantes et al 2020a, 2020b). We here corroborate these findings and add that hybrid males might also be present and reproduce with both parental species. Therefore, previous results that assumed that the parental F1 individual was always female might be inaccurate.

Another interesting fact concerns the new haplotypes found in hybrid individuals, which fall in between two parent species when the Networks are analysed. The presence of shared SNPs could be explained by the retention of the ancestral allele, which was present in the last common ancestor of the species. However, incomplete lineage sorting (ILS) where ancestral polymorphisms are retained through speciation must be considered as an alternative explanation for shared genetic variation. ILS is particularly relevant for species with large effective population sizes and long generation times, both characteristics of sea turtles. Our study's reliance on four nuclear loci limits our ability to fully distinguish between ILS and recent introgression, as genome-scale data would provide greater power to differentiate these processes. Nevertheless, the combination of multiple diagnostic SNPs across our loci provides robust hybrid identification for conservation purposes, while future genomic studies could help disentangle the relative contributions of ancestral polymorphism versus ongoing gene flow in the Abrolhos population.

Although it is possible to identify outbreeding depression in F1 generation in many purebred species (Ralls et al 2013), the highly conserved genome of marine turtles (Bentley et al 2022) and their historical hybridisation background (Vilaça et al 2022) might have braked the negative consequences of mixing genomes that have been diverging during the last 29 million years (Duchene et al 2012). As seen in previous studies that indicate that there is no significant difference in fitness between hybrids and nonhybrids (Soares et al 2017, 2018). This might be partially explained by the fact all extant sea turtles have the same chromosomal number (2n = 56) and there is strong synteny between chromosomes within Testudines, but particularly between Cheloniidae species (Lee et al 2018, Vilaça et al 2021, Bentley et al 2022). However, previous evidence suggests that F1 hybrid nests in Abrolhos have lower fitness compared to non-hybrids, as indicated by reduced hatchling success (Arantes et al 2020a). This may be due to recombination between chromosomes of different species, which occurs from the F2 generation onward and can break coadapted gene complexes, potentially impacting individual fitness (Whitlock et al 2013).

To date, F2 hybrids have not been confirmed among sampled reproductive adults (Vilaça et al 2022), though this may reflect sampling limitations rather than absolute reproductive failure, as adult turtles and particularly males are challenging to sample and genotype. In this context, F2 individuals may not reach adulthood due to genetic incompatibilities, such as Dobzhansky-Muller incompatibilities (DMI) (Dagilis et al 2019). DMI implies that there could be negative epistatic interactions with different alleles that are found together in the F2 hybrid, leading to lethal combinations or poorly fit individuals (Dobzhansky 1937). Therefore, based on the natural history of the clade and its ancient hybridisation processes (Vilaça et al 2022), although historical hybridisation has occurred, the low fitness of F2 hybrids observed today may be related to a currently higher rate of hybridisation than seen in the past. Alternatively, it could be also due to more efficient speciation barriers (reinforcement) acquired during the ongoing speciation process (Butlin and Smadja 2018). We propose that meiotic recombination, which disrupts favourable allelic combinations and leads to reduced fitness —a phenomenon known as outbreeding depression— could be a key factor contributing to the absence of F2 adults on Brazilian beaches.

The predominance of F2 hatchlings in our sample does not necessarily indicate F2 adult reproduction. Our proposed crossings (fig. 1) most parsimoniously explain these offspring as products of F1 × F1 or F1 × purebred matings, though F2 adult reproduction cannot be excluded without direct parental genotyping. The absence of confirmed F2 adults (Vilaça et al 2022) may reflect sampling limitations rather than reproductive failure.

Based on the individuals' analyses, it was possible to infer how hybrids behave regarding mating choice. Based on the inferred parental genotypes, hybrids appear to mate most frequently with *C. caretta* individuals (47.5 % of hybrid matings), followed by *E. imbricata* (40.7 %), and other hybrids (11.9 %). However, these proportions likely reflect the relative abundance of potential mates rather than active mate preference. Loggerheads are the most common species in the region, followed by hawksbills (IUCN 2021), and the mating frequencies we observe closely mirror these population densities. This pattern suggests that hybrid turtles may mate opportunistically with available individuals regardless of species or hybrid status, a behaviour that could be driven by reduced conspecific availability in declining populations. Alternatively, hybrids may be unable to discriminate between species during mating, or species recognition cues may be disrupted in hybrid individuals. Field observations of mating behaviour would be necessary to distinguish between these hypotheses.

Although *C. caretta* and *E. imbricata* species diverged approximately 29 million years ago (Duchene et al 2012), both exhibit low population genetic diversity and strong genetic structure between nesting sites (Simões et al 2021). This pattern has been potentially attributed to their slow metabolism and evolutionary rate, strong purifying selection maintaining their specialized morphology, or philopatric behaviour. However, despite this low diversity, crossings between F1 hybrids that produce F2 individuals can disrupt species-specific allele combinations through meiotic recombination. These disruptions can introduce potentially deleterious genetic combinations, decreasing the fitness of local populations and creating significant challenges for conservation efforts.

Nonetheless, there is a need for further research on hybrid fitness, mating preferences, and how hybridisation might alter population structures and genetic diversity. Expanding this research to include genomic studies could reveal adaptive traits that hybrids might carry and clarify whether hybridisation is a response to environmental pressures or population dynamics unique to north-eastern Brazil.

Despite the high prevalence of F1 hybrid females in north-eastern Brazilian beaches (Marcovaldi et al 1997), outbreeding depression appears to affect sea turtle populations in Abrolhos. The fact that F2 adults have not been found so far, both in Abrolhos and other locations in Brazil, may indicate that these hatchlings do not reach adulthood. Thus, although F1 hybrids can reproduce, their offspring (F2 hybrids) may suffer from outbreeding depression in survival and/or reproduction due to genetic incompatibilities.

Abrolhos hatchlings are mostly pure *C. caretta*, and some new haplotypes have been identified in certain individuals and nests. Hybrids are frequently observed, and notably, no 'pure' *E. imbricata* offspring were detected in our study. Even nests that appear to contain pure *Ei* individuals also contain *Cc* alleles, indicating that these seemingly 'pure' individuals are likely 'false pure' —not detected as introgressed due to the limited number of markers used (as in nest AB46 in fig. 1D). This highlights the necessity for further studies employing more extensive genomic regions to accurately identify introgression patterns.

The analysis of multiple siblings allowed the identification of the parental genotypes that originated them, which was not possible with the genomic analysis of one individual per nest in a previous study (Arantes et al 2020a). Most hybrid offspring studied here are at least F2, but there could also be hybrid F1 individuals. If the sampling carried out in this research is consistent with the true composition of the Abrolhos population, 42 % of the hatchlings should be hybrids, the majority being F2. However, if the F2 hybrids are suffering outbreeding depression and do not reach adulthood or reproduce, a significant portion of the Abrolhos offspring may be inviable, representing a potentially serious conservation threat. This concern is supported by previous studies demonstrating that hybrid nests in this population exhibit significantly lower hatching success compared to pure *C. caretta* nests (Arantes et al 2020a). Arantes et al (2020a) documented reduced emergence rates and higher embryonic mortality in hybrid clutches, providing quantitative evidence that hybridization negatively impacts reproductive output in the Abrolhos population. Combined with our finding that the majority of dead hatchlings analysed were F2 hybrids, these results suggest that outbreeding depression may be limiting population viability and warrant continued monitoring and assessment of conservation strategies.

Our comprehensive genetic analysis has expanded the molecular toolkit for identifying and characterizing hybridisation between *C. caretta* and *E. imbricata* sea turtles, particularly from poor quality samples as these decaying tissues from dead hatchlings. The discovery of novel species-specific SNPs and previously undescribed haplotypes enhances our understanding of the complex genetic diversity in this hybridisation zone. These genetic insights provide information for conservation management, enabling more precise identification of purebred and hybrid individuals, assessment of hybridisation extent, and development of targeted protection strategies for these threatened marine turtle species in areas where interbreeding occurs. These findings collectively strengthen our capacity to monitor hybridisation dynamics and implement targeted conservation strategies for these threatened marine turtle species in areas where interspecific breeding occurs.

Our study has some limitations. First, we analysed only four nuclear loci, which limits our power to detect introgression and distinguish F1 from F2 individuals with complete certainty. Some individuals classified as F1 may actually be F2 with undetected introgressed alleles (false negatives), and conversely, some F2 individuals may appear purebred due to Mendelian sampling (12.5 % probability with four markers). Second, we cannot directly genotype parents, requiring us to infer parental genotypes from offspring patterns and alternative crossing schemes may explain some nest compositions. Third, all samples came from deceased hatchlings, preventing us from assessing fitness differences between crossing types through survival analysis. Finally, we cannot determine whether observed mating patterns reflect active mate preferences or simply opportunistic mating driven by relative species abundances.

Future studies should prioritize genotyping living hatchlings and adult individuals, implementing molecular sexing techniques to investigate potential sex-biased mortality patterns in hybrids, and conducting long-term monitoring to assess the survival and reproductive success of hybrid offspring through multiple life stages, which will be essential for understanding the evolutionary dynamics and conservation implications of hybridization in sea turtle populations.

This study provides the first genetic evidence that hybrid male sea turtles are fertile and actively reproducing in the Abrolhos Archipelago. By analysing multiple siblings per nest with species-specific nuclear markers, we demonstrated that F1 hybrids of both sexes can mate with each other, with purebred individuals of both parental species, and potentially across multiple hybrid generations. The discovery of fertile hybrid males fundamentally changes our understanding of gene flow dynamics in this system, revealing bidirectional introgression rather than female-mediated gene flow alone. With 42 % of hatchlings showing hybrid ancestry (predominantly F2), and evidence of multiple paternity involving hybrids and both parental species, the Abrolhos population exhibits complex and ongoing genetic admixture. The absence of confirmed F2 adults despite abundant F2 hatchlings suggests potential outbreeding depression, raising serious conservation concerns about population viability. These findings underscore the urgent need for long-term monitoring, assessment of hybrid fitness across life stages, and development of targeted conservation strategies for this unique hybridization hotspot where two threatened sea turtle species are undergoing rapid genetic admixture.

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Author contributions
FRS was responsible for conveying and designing the analysis and discussing results. LSA designed the primers used, performed the wet lab work and discussed the results. JFM performed the wet lab work and data analysis and discussed the results. All authors wrote the manuscript and approved the final version.

Conflicts of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability
Genbank accession numbers can be found in the Supplementary Material and are under BioProject PRJNA641778 with sample-specific accession numbers 39212780 - 39212792.

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Supplementary material

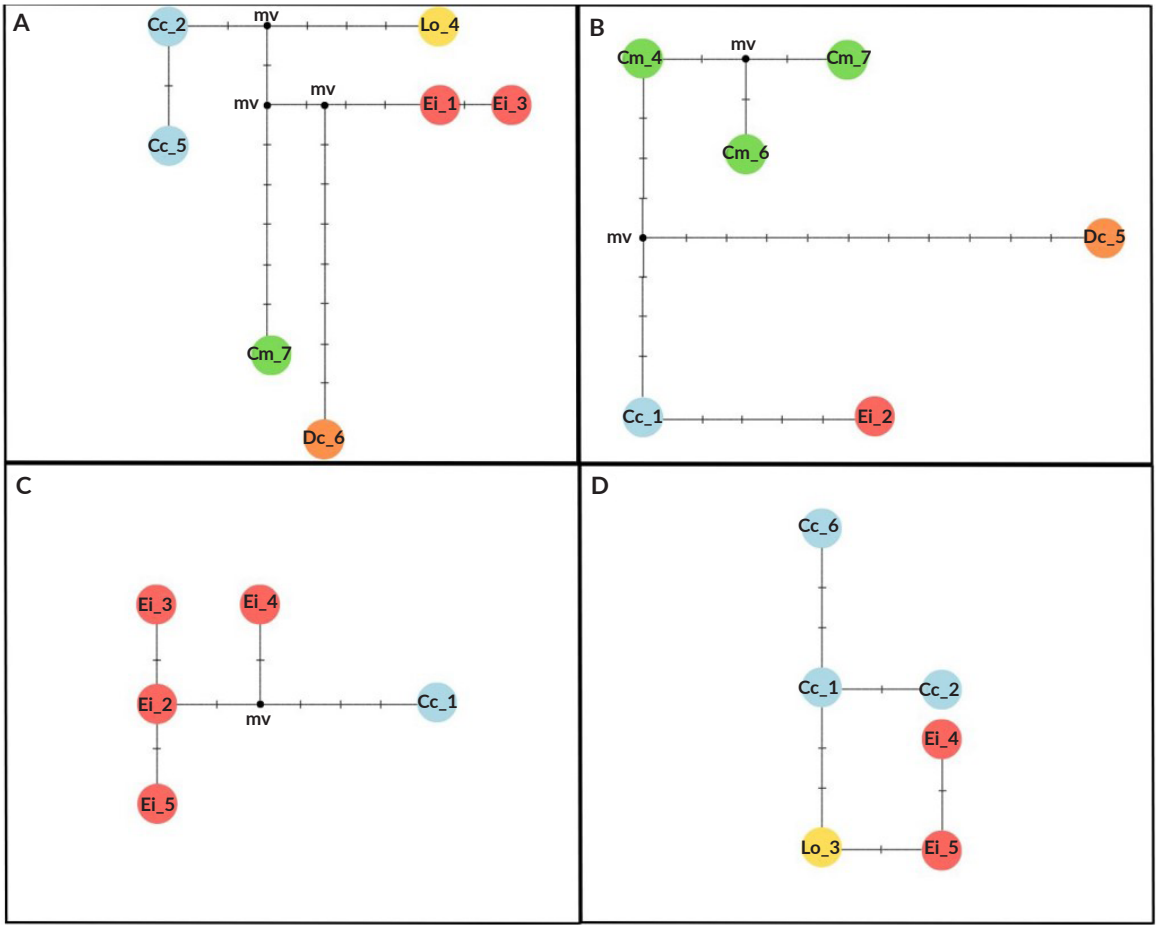

Fig. S1. Network of haplotypes for all sea turtle species that nest along the Brazilian coast, based on loci previously characterized by Arantes et al (2020b). The size of the circles represents the number of alleles in each haplotype. Since all individuals are homozygotes, and there is only one individual per haplotype, all circles are the same size. Panels show loci A) 3061, B) 64188, C) 76958, and D) 856. Each dash represents one mutational step between haplotypes, and 'mv' denotes a hypothetical, unsampled, or ancestral haplotype inferred by the program to connect observed haplotypes. Species are indicated by colour: *Caretta caretta* (**Cc**, blue), *Eretmochelys imbricata* (**Ei**, red), *Chelonia mydas* (**Cm**, green), *Dermochelys coriacea* (**Dc**, orange), and *Lepidochelys olivacea* (**Lo**, yellow). When multiple haplotypes occur within the same species, they remain clustered more closely to each other than to haplotypes from other species, further demonstrating strong interspecific genetic separation. This is particularly important between *C. caretta* and *E. imbricata*, the focal species of this study.

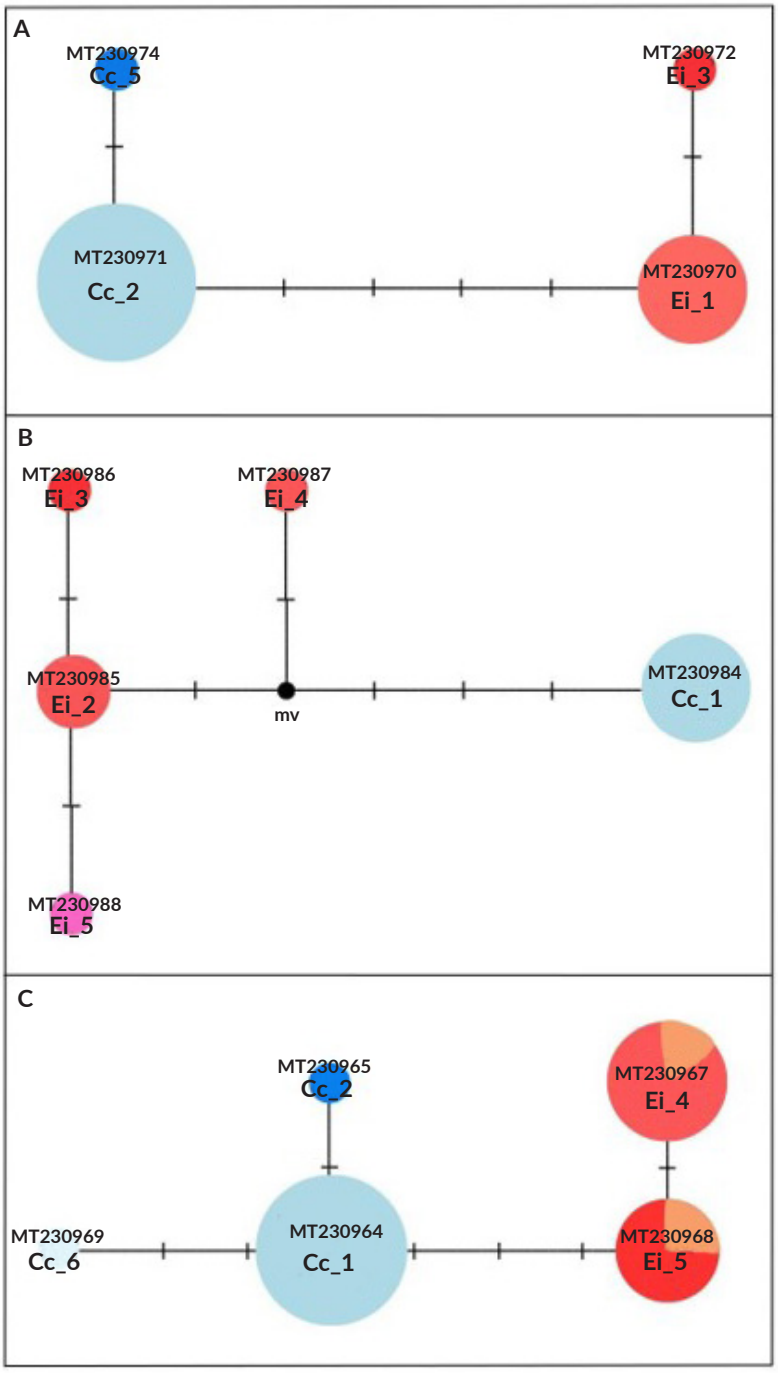

Fig. S2. Network representation of genetic relationships among pure *C. caretta* (blue tones) and *E. imbricata* (red tones) individuals from Brazil, showing that pure individuals consistently display species-specific SNPs/haplotypes. The size of the circles represents the number of alleles in each haplotype. Individuals analysed and their sampling localities are listed in table S3. Panels show loci A) 3061, B) 76958, and C) 856. The 64188 region was not included because it contains only two haplotypes (one for *C. caretta* and one for *E. imbricata*), preventing meaningful network visualization; all individuals are homozygous at this locus, and most (including all Abrolhos samples) cluster with haplotype Cc_Lo_1, shared by *C. caretta* and *Lepidochelys olivacea*, while only R0206 and R0028 cluster with Ei_2 (data not shown). At 3061 (A), all Abrolhos individuals are homozygous for Cc_2, and individuals from other localities (also homozygous) are distributed across additional *C. caretta* haplotypes. At 76958 (B), all Abrolhos individuals fall within haplotype Cc_1, whereas individuals from other regions cluster in Ei_2, and all are homozygous. At 856 (C), all Abrolhos individuals are homozygous for Cc_1; individuals R0592 and R0176 are heterozygous for *E. imbricata*, each carrying alleles Ei_4 and Ei_5 (in orange), while all remaining individuals are homozygous.

Table S1. Primer sequences, amplicon size and sequence, PCR annealing temperature and diagnostic sites of all loci analysed: 856, 3061, 64188 and 76958.

Locus	Forward primer sequence 5'- 3'	Reverse primer sequence 5'- 3'	Amplicon size (bp)	Amplicon sequence	Annealing temperature	Number of diagnostic sites		
						Cc x Ei	Cc x Lo	Ei x Lo
856	CCCTGACCTTTGTGCAGGCA	TGAGGGTTAGATTGTGTCTACCGT	297	TATGGTCCCTGACCTTTGTGCAGGCACACAGGGTGATGAG AGTGCCAATACGTTCCTATGTGTCCCACACAAGGGCAAATA ACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACTGCTA TTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGG CAAAGATGGAGGAGGAACCCTGCCAGGCAGAGCAGGCTG AACCATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACT CCTGGCAGCTGTGGGAAGGATAAACCTTCCTTAAAGTCTCA GTGGAGTAGGGAGCTCCACAGCAGCCCCAAAGCATGACTG TAACGGTAGACACAATCTAACCCTCAGACACTCACTAATGG GACTGAGGCATTTGGTTAATT	59°C	3	2	1
3061	GTGCCCACACACCACAAGAT	CATCACATTTATGTTTGTGTATCTGCA	334	TATGGTTTGTGCCCACACACCACAAGATATGGCAGTTATAAT GATGGCTGGGTAAGTAGTGTCCATCGTGGCTAGGCTAGTGC TTGGTTAGTAACCATGAAAGTTAAGTGTTTTGTCCTTGCCAG AGGCCTAGGCTGGCACATGTCATCCTGTAATCAGTCTGTCAC AGCTTTTTGAACTGGGAGCAGGACCCTCACCTGTGTCTGTG TAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGT TGCCTCTTATGCTTATCTTTGTGCATGTTCTCCGGGCACTCAA ACCCCTGCTGAGTCAAGAAGGAGAACTGTCAAGTGCACTGT TACAAACTGGATTAGGCACATGGTAGGTGCAGATACACAAA CATAAATGTGATGAAAGAAAAATT	56°C	4	4	6
64188	GGGACCATATTCTTCCCTGCACT	CTTGTTCCTTCCCAAGTGGAGT	349	TATGAGGGACCATATTCTTCCCTGCACTGTGGTGGCTTTATG CCACATTGATGCTCAAAGCAGACATAAAGCCAGAGTAGCTG GCTAGTGGAGGATGATCTCAGGGTAGGAGACTCTTCTGATG GGGAAGTGTCAGAGTAACAGTTCTTCCATCTCCTTGTGATCA ATGGTGGAGAAGGAAGGATGGTCTGTTGGGTAGAGCACTAG AATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAG TTACATCACCAAACAGTTGTCAAAGTTTGTATGTCACTTGGG TGTATGCATACTTGTCTGCTTGCGTCAATGCTGCACATACTCA CCAGGACTGCCCATGTCAATGCAAAGTACTCCACTTGGGAA GGAACAAGTAATT	58°C	4	0	4
76958	CAAGCACCCTGCCAGAGGTC	ACCTGCATTCTGCCATATATTTCATGT	330	TATGTTCCTTCATGCTTCAAGCACCCTGCCAGAGGTCATGCA TCCATGCCGATGACAGTTTCTGCTCGATAACGATCCAAAGCA AATGCCACTAGCAGAAGGATCTTGTTTGGTGGTTTGGGTTCT GTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAA AGCATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCA CTTCAGAGTCTTAAACCGTGGGTCGAGTGCTGTAGCTATCTT TAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCT GCAGCAAAAGTGTACTTAAAATGAACAACGTGCTGTCATCAT CCAGGACTACTTAACATGAAATATATGGCAGAATGCAGGTAA AATAGAGCCAGAGACAAACAATT	64°C	3	-	-

Table S2. GenBank accession numbers for species-specific haplotypes and sequences of all loci analyzed are 856, 3061, 64188, and 76958, referring to all sea turtle species that nest on Brazilian beaches. Species abbreviations are as follows: Ei: *Eretmochelys imbricata*; Cc: *Caretta caretta*; Lo: *Lepidochelys olivacea*; Dm: *Dermochelys coriacea*; and Cm: *Chelonia mydas*. The numbers following each species abbreviation correspond to the haplotype identifiers.

Genetic marker	Haplotype	Species	GenBank acession number	Sequence
3061	Ei1	*Eretmochelys imbricata*	MT230970	TATGGTTTGTGCCCACACACCACAAGATATGGCAGTTATAATGATGGCTGGGTAAGTAGTGTCCATCGTGGCTAGGCTAGTGCTTGGT TAGTAACCATGAAAGTTAAGTGTTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATCCTGTAATCAGTCTGTCACAGCTTTTTG AACTGGGAGCAGGACCCTCACCTGTGTCTGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTATGCT TATCTTTGTGCATGTTCTCCGGGCACTCAAACCCCTGCTGAGTCAAGAAGGAGAACTGTCAAGTGCACTGTTACAAACTGGATTAGG CACATGGTAGGTGCAGATACACAAACATAAATGTGATGAAAGAAAAATT
	Cc2	*Caretta caretta*	MT230971	ACAAGATATGGCAGTTATAATGATGGCTGGGTAAGTAGTGTCCATCGTGGCTAGGCTAGTGCTTGGTTAGTAACCATGAAAGTTAAGT GTTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATCCTGTAATCAGTCTGTCACAGGTTTTTGAACTGGGAGCAGGACCCTCA CCTGTGTCTGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTATGCTTATCTTTGTGCATGTTCTCCAG GCACTCAAACCCCTGCTGAGTCAAGAAGGAGAGCTGTCAAGTGCACTGTTACAAACTGGATTAGGCGCAT
	Ei3	*Eretmochelys imbricata*	MT230972	ACAAGATATGGCAGTTATAATGATGGCTGGGTAAGTAGTGTCCATCGTGGCTAGGCTAGTGCTTGATTAGTAACCATGAAAGTTAAGT GTTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATCCTGTAATCAGTCTGTCACAGCTTTTTGAACTGGGAGCAGGACCCTCA CCTGTGTCTGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGTTGCCTCTTATGCTTATCTTTGTGCATGTTCTCCGGG CACTCAAACCCCTGCTGAGTCAAGAAGGAGAACTGTCAAGTGCACTGTTACAAACTGGATTAGGCACAT
	Lo4	*Lepidochelys olivacea*	MT230973	ACAAGATGTGGCAGTTATAATGATGGCTGGGTAAGTAGTGTCCATCGTGGCTAGGCTAGTGTTTGGTTCGTAACCATGAAAGTTAAGT GTTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATCCTGTAATCAGTCTGTCACAGCTTTTTGAACTGGGAGCAGGACCCTCA CCTGTGTCTGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTATGCTTATCTTTGTGCATGTTCTCCAG GCACTCAAACCCCTGCTGAGTCAAGAAGGAGAGCTGTCAAGTGCACTGTTACAAACTGGATTAGGCGCAT
	Cc5	*Caretta caretta*	MT230974	ACAAGATATGGCAGTTATAATGATGGCTGGGTAAGTAGTGTCCATCGTGGCTAGGCTAGTGCTTGGTTAGTAACCATGAAAGTTAAGT GTTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATTCTGTAATCAGTCTGTCACAGGTTTTTGAACTGGGAGCAGGACCCTCA CCTGTGTCTGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTATGCTTATCTTTGTGCATGTTCTCCAG GCACTCAAACCCCTGCTGAGTCAAGAAGGAGAGCTGTCAAGTGCACTGTTACAAACTGGATTAGGCGCAT
	Dc6	*Dermochelys coriacea*	MT230975	ACAAGATATGGCAGTTATAATGATGGCTGGGTAACTAGTGTCCATTGTGGCTAGGCTAGTGCGTGGTTAGTAACCATGAAAGTTAAGT GTTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATCCTGAAATCAGTCTGTCGCAGCTTTTTGAACTGGGAGCAGGACCCTCA CCTGTTTCTGTGTAACTGGTTTGGGTGGTCTTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTATGCTTATCTTTGTGC ATGTTCTCCAG GCACTCAAACCCCTGCTGAGTCAAGAAGAACTGTCAAGTGCACTGTTACAAACTGGATTAGGCACAT
	Cm7	*Chelonia mydas*	MT230976	ACAAGATATAGCAGTTATAATGATGGCTGGTAAGTAGCGTCCATCGTGGCTAGGCTAGTGCGTGGTTAGTAACCATGAAAGTTAAGTG TTTTGTCCTTGCCAGAGGCCTAGGCTGGCACATGTCATCCTGTAATCAGTCTGTCACAGCTTTTTGAACTGGAAGCAGGACCCTCACC TGTGTCTGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTACGCTTATCTTTGTGCATGTTCTCCAGGC ACTCAAACCCCTGCTGAGTC AAGAAGGAGAACTGTCAAGTGCACTGTTACAAACTGGATTAGGCGCAT
64188	Cc1	*Caretta caretta*	MT230977	CTGCACTGTGGTGGCTTTATGCCACATTGATGCTCAAAGCAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGATCTCAGGGTA GGAGACTCTTCTGATGGGGAAGTGTCAGAGTAACAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGATGGTCTGTTGG GTAGAGCACTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACATCACCAAACAGTTGTCAAAGTTTGTATGTC ACTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGCTGCACATACTCACCAGGACTGCCCATGTCAATGCAAAGTACTCCACTTG
	Ei2	*Eretmochelys imbricata*	MT230978	CTGCACTGTGGTGGCTTTATGCCACATTGATGCTCAAAGCAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGATCGCAGGGTA GGAGACTCTTCTGATGGGGAAGTGTCAGAGTAACAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGACGGTCTGTTGG GTAGAGCGCTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACATCACCAAACAGTTGTCAAAGTTTGTGTGT CACTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGCTGCAC ATACTCACCAGGACTGCCCATGTCAATGCAAAGTACTCCACTTG
	Cm4	*Chelonia mydas*	MT230980	CTGCACTGTGGTGGCTTTATGCCACACTGATGCTCAAAGCAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGGTCTCAGGGTA GGAGAATCTTCTGGTGGGGAAGTGTCAGAGTAATAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGATGGTCTGTTGG GTAGAGCACTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACACCACCAAACAGTTGTCAAAGTTTGTATGTC ACTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGC TGCACATACTCACCAGGACTGCCTGTGTCAATGCAAAGTACTCCACTTG

Table S2. (Cont.)

Genetic marker	Haplotype	Species	GenBank acession number	Sequence
	Dc5	*Dermochelys coriacea*	MT230981	CTGCACTGTGGTGGCTTTATGCCACATTGATGCTCAAAGCAGACGTAAAGCCAGAGTAGCTGGCTAATGGAGGATGATCTCAGGGTA GGAGAATCTTCTGATGGCCATGTTAGCACGACAAACTGTGGTCAACGCAAGTTACATCACCAGACAGTTGTCAAAGTTTGTATGTCAC TTGGGTGCATGCATACTTGTCTGCTTGCATCAATGCTGCACATACTCACCAGGACTGCCTGTGTCAATGCAAAGTACTCCACTTG
	Cm6	*Chelonia mydas*	MT230982	CTGCACTGTGGTGGCTTTATGCCACACTGATGCTCAAAGCAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGGTCTCAGGGTA GGAGAATCTTCTGATGGGGAAGTGTCAGAGTAATAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGATGGTCTGTTGGG TAGAGCACTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACATCACCAAACAGTTGTCAAAGTTTGTATGTCA CTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGCTGCACATACTCACCAGGACTGCCTGTGTCAATGCAAAGTACTCCACTTG
	Cm7	*Chelonia mydas*	MT230983	CTGCACTGTGGTGGCTTTATGCCACACTGATGCTCAAAGCAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGGTCTCAGGGTA GGAGAATCTTCTGATGGGGAAGTGTCAGAGTAATAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGATGGTCTGTTGGG TAGAGCACTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACACCACCAAACAGTTGTCAAAGTTTGTATGTCA CTTGGGTGTATGCATACTTGTCTACTTGCGTCAATGCTGCACATACTCACCAGGACTGCCTGTGTCAATGCAAAGTACTCCACTTG
76958	Cc1	*Caretta caretta*	MT230984	GGTCATGCATCCATGCCGATGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGATCTTGTTTGGTGGTTTGG GTTCTGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCAC TTCAGAGTCTTAAACCGTGGGTCGAGTGCTGTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGC AAAAGTGTACTTAAAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATATG
	Ei2	*Eretmochelys imbricata*	MT230985	GGTCATGCATCCATGCCGTTGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGTTCTTGTTTGGTGGTTTGG GTTATGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCAC TTCAGAGTCTTAAACCGTGGGTTGAGTGCTGTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGC AAAGTGTACTTAAAATGAACAACGTGCTGTATCATCCAGGACTACTTAACATGAAATATATG
	Ei3	*Eretmochelys imbricata*	MT230986	GGTCATGCATCCATGCCGTTGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGTTCTTGTTTGGTGGTTTGG GTTATGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCAC TTCAGAGTCTTAAACCGTGGGTTGAGTGCTGTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATTTGCAGCA AAAGTGTACTTAAAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATATG
	Ei4	*Eretmochelys imbricata*	MT230987	GGTCATGCATCCATGCCGATGACAGTTTCTGCTCGATAATGATCCAAAGCAAATGCCACTAGCAGAAGGTTCTTGTTTGGTGGTTTGG GTTATGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCAC TTCAGAGTCTTAAACCGTGGGTTGAGTGCTGTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCA AAAGTGTACTTAAAATGAACAACGTGCTGTATCATCCAGGACTACTTAACATGAAATATATG
	Ei5	*Eretmochelys imbricata*	MT230988	GGTCATGCATCCATGCCGTTGACAGTTTCTGCTCGATAACGATCCAAACCAAATGCCACTAGCAGAAGGTTCTTGTTTGGTGGTTTGG GTTATGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCAC TTCAGAGTCTTAAACCGTGGGTTGAGTGCTGTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCA AAAGTGTACTTAAAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATATG
856	Cc1	*Caretta caretta*	MT230964	GGTGATGAGAGTGCCAATACGTTCCTATGTGTCCCACACAAGGGCAAATAACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACTG CTATTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACCCTGCCAGGCAGAGCAGGCTGAAC CATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACTCCTGGCAGCTGTGGGAAGGATAAACCTTCCTTAAAGTCTCAGTGGAGTAG GGAGCTCCACAGCAGCCCCAAAGCATGACTGTAA
	Cc2	*Caretta caretta*	MT230965	GGGTGATGAGAGTGCCAATACGTTCCTATGTGTCCCACACAAGGGCAAACAACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACT GCTATTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACCCTGCCAGGCAGAGCAGGCTGAA CCATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACTCCTGGCAGCTGTGGGAAGGATAAACCTTCCTTAAAGTCTCAGTGGAGTA GGGAGCTCCACAGCAGCCCCAAAGCATGACTGTAA
	Lo3	*Lepidochelys olivacea*	MT230966	GGGTGATGAGAGTGCCAATACGTTCCTATGTGTCCCACACAAGGGCAAATAACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACT GCTATTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACTCTGCCAGGCAGAGCAGGCTGCA CCATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACTCCTGGCAGCTGTGGGAAGGATAAACCTTCCTTAAAGTCTCAGTGGAGTA GGGAGCTCCACAGCAGCCCCAAAGCATGACTGTAA

Table S2. (Cont.)

Genetic marker	Haplotype	Species	GenBank acession number	Sequence
	Ei4	*Eretmochelys imbricata*	MT230967	GGGTGATGAGAGTGCCAATACGTTCCTGTGTGTCCCACACAAGGGCAAATAACAGCCTTAGTCCCCGAACTCAAAGGAGCACTGACT GCTATTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACTCTGCCAGGCAGAGCAGGCTGCAC CATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACTCCTGGCAGCTGTGGGAAGGATAAACCTTCCTTAAAGTCTCAGTGGAGTAG GGAGCTCCACAGCAGCCCCAAAGCATGACTGTAA
	Ei5	*Eretmochelys imbricata*	MT230968	GGGTGATGAGAGTGCCAATACGTTCCTGTGTGTCCCACACAAGGGCAAATAACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACT GCTATTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACTCTGCCAGGCAGAGCAGGCTGCAC CATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACTCCTGGCAGCTTGGGAAGGATAAACCTTCCTTAAAGTCTCAGTGGAGTAGG GAGCTCCACAGCAGCCCCAAAGCATGACTGTAA
	Cc6	*Caretta caretta*	MT230969	GGGTGATGAGAGTGCCAATACGTTCCTATGTGTCCCACACAAGGGCAAATAACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACT GCTATTTTTGTGTGTCCCCAGAGGTGAATCTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACCCTGCCAGGCAGAGCAGGCTGAA CCATCCTTAAGTTCAGTGTACTCTACATTTCTGCTGCACTCCTGGCAGCTGTGGGAAGGATAAACCTTCCTTAAAGTCTCAGTGGAGTA GGGAGCTGTACAGCAGCCCCAAAGCATGACTGTAA

Table S3. Individual samples per locality and genomic loci sequenced. NI, indicates that the locality could not be identified; x, indicates successful sequencing of an individual for a given locus.

			Genomic loci			
Individuals	**Characteristics**	**Location**	**3061**	**64188**	**76958**	**856**
Ind_Cc1	*Caretta caretta*	NI	x	x	x	x
Ind_Cc2	*Caretta caretta*	NI	x	x	x	x
Ind_Cc3	*Caretta caretta*	NI	x	x	x	x
Ind_Cc4	*Caretta caretta*	NI	x	x	x	x
Ind_Cc5	*Caretta caretta*	NI	x	x	x	x
AB9F2	Tested hatchling	Abrolhos	x	x	x	x
AB12F1	Tested hatchling	Abrolhos	x	x	x	x
AB13F2	Tested hatchling	Abrolhos	x	x	x	x
AB16F3	Tested hatchling	Abrolhos	x	x	x	x
AB18F4	Tested hatchling	Abrolhos	x	x	x	x
AB20F1	Tested hatchling	Abrolhos	x	x	x	x
AB21F1	Tested hatchling	Abrolhos	x	x	x	x
AB23F1	Tested hatchling	Abrolhos	x	x	x	x
AB25F1	Tested hatchling	Abrolhos	x	x	x	x
AB36F2	Tested hatchling	Abrolhos	x	x	x	x
AB38F2	Tested hatchling	Abrolhos				x
R0028	*Eretmochelys imbricata*	Atol das Rocas	x	x	x	
R0102	*Eretmochelys imbricata*	Fernando de Noronha	x	x	x	x
R0176	*Eretmochelys imbricata*	Bahia	x		x	x
R0206	*Eretmochelys imbricata*	Fernando de Noronha		x		
R0337	*Eretmochelys imbricata*	Elevação do Rio Grande	x	x	x	x
R0592	*Eretmochelys imbricata*	Rio Grande no Norte	x		x	x

Table S4. GenBank accession numbers for the new haplotypes identified in this study, the parental species showing the closest genetic affinity to each haplotype, the individuals in which each haplotype was detected, and the corresponding sequences.

Genetic marker	Haplotype	Parental species	Individual	GenBank acessionn number	Sequence
3061	Cc8_3061	*Caretta caretta*	AB11F6	SAMN39212780	GCTAGGCTAGTGCTTGGTTAGTAACCATGAAAGTTAAGTGTTTTGTCCTTGCCAGAGGCCTAGGCTG GCACATGTCATCCTGTAATCAGTCTGTCACAGGTTTTTGAACTGGGAGCAGGACCCTCACCTGTGTC TGTGTAACTGGTTTGGGTGGTCCTCCCTCCTTCCAGTTGGTGTGGTTGCCTCTTATGCTTATCTTTGT GCATGTTCTCCR GGCACTCAAACCCCTGCTGAGTCAAGAAGGAGAGCTGTCAAGTGCACTGTTAC AAACTGGATTAGGCR
64188	Cc8_64188	*Caretta caretta*	AB38F8	SAMN39212782	CAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGATCTCATGGGTAGGAGACTCTTCTGATGG GGAAGTGTCAGAGTAACAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGATGGTCTG TTGGGTAGAGCACTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACATCAC CAAACAGTTGTCAAAGTTTGTATGTCACTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGCT
	CcxEi9_64188	*Caretta caretta* x *Eretmochelys imbricata*	AB43F5	SAMN39212783	AGCAGACATAAAGCCAGAGTAGCTGGCTAGTGGAGGATGATCTCANGGGTAGGAGACTCTTCTGA TGGGGAAGTGTCAGAGTAACAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGAYGGT CTGTTGGGTAGAGCRCTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACATC ACCAAACAGTTGTCAAAGTTTGTRTGTCACTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGCT
	CcxEi10_64188	*Caretta caretta* x *Eretmochelys imbricata*	64188CcxEi Hybrids	SAMN39212784	CAGAGTAGCTGGCTAGTGGAGGATGATCKCANGGGTAGGAGACTCTTCTGATGGGGAAGTGTCAG AGTAACAGTTCTTCCATCTCCTTGTGATCAATGGTGGAGAAGGAAGGAYGGTCTGTTGGGTAGAGCR CTAGAATAAGGCCATGTTAGCACTACAAACTGTGGTCAACGCAAGTTACATCACCAAACAGTTGTCA AAGTTTGTRTGTCACTTGGGTGTATGCATACTTGTCTGCTTGCGTCAATGCT
76958	CcxEi6_76958	*Caretta caretta* x *Eretmochelys imbricata*	AB11F1	SAMN39212785	GCATCCATGCCGATGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGWTC TTGTTTGGTGGTTTGGGTTMTGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCAT GCTCCACACCACATCCTGCTCAGATTTTGGAAGGCACTTCAGAGTCTTAAACCGTGGGTCGAGTGCT GTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCAAAAGTGTAC TTAAAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATATG
	Cc7_76958	*Caretta caretta*	AB20	SAMN39212786	GCATCCATGCCGATGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGATCT TGTTTGGTGGTTTGGGTTCTGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCATG CTCCACACCACATCCTGCTCAGATTTTGGAAGGCACTTCAGAGTCTTAAACCGTGGRTCGAGTGCTG TAGCTATCWTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCAAAAGTGTAC TTAAAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATATG
	Cc8_76958	*Caretta caretta*	AB21F1	SAMN39212787	GCATCCATGCCGATGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGATCT TGTTTGGTGGTTTGGGTTMTGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGCAT GCTCCACACCACATCCTGCTCAGATTTTGGAAGGCACTTCAGAGTCTTAAACCGTGGGTCGAGTGCT GTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCAAAAGTGTA CTTAAAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATA
	CcxEi9_76958	*Caretta caretta* x *Eretmochelys imbricata*	76958CcxEi Hybrids	SAMN39212788	GCATCCATGCCGWTGACAGTTTCTGCTCGATAACGATCCAAAGCAAATGCCACTAGCAGAAGGWT CTTGTTTGGTGGTTTGGGTTMTGTAGTTTCTGCATCGGAGTGTTGCTCTTTTAAGACATCTGAAAGC ATGCTCCACACCACATCCTGCTCAGATTTTGGAAGGCACTTCAGAGTCTTAAACCGTGGGTYGAGTG CTGTAGCTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCAAAAGTGT ACTATCTTTAGAAATCTTACATTGGTACCTTCTTTGCGTTTTGCCAAATCTGCAGCAAAAGTGTACTTA AAATGAACAACGTGCTGTCATCATCCAGGACTACTTAACATGAAATATA

Table S4. (Cont.)

Genetic marker	Haplotype	Parental species	Individual	GenBank acessionn number	Sequence
856	CcxEi7_856	*Caretta caretta* x *Eretmochelys imbricata*	AB17	SAMN39212789	AACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACTGCTATTTTTGTGTGTCCCCAGAGGTGAAT CTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACYCTGCCAGGCAGAGCAGGCTGMACCATCCT TAAGTTCAGTGTACTCTACATTTCT
	Cc8_856	*Caretta caretta*	AB41F4	SAMN39212790	AACAGCCTTAGTCCCCGAACTCAAAGGAGCACTGACTGCTATTTTTGTGTGTCCCCAGAGGTGAAT CTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACYCTGCCAGGCAGAGCAGGCTGMACCATCCT TAAGTTCAGTGTACTCTACATTTCT
	Cc9_856	*Caretta caretta*	AB46F6andAB8	SAMN39212791	AACAGCCTTAGTCCCCGAGCTCAAAGGAGCACTGACTGCTATTTTTGTGTGTCCCCAGAGGTGAAT CTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACYCTGCCAGGCAGAGCAGGCTGAACCATCCTT AAGTTCAGTGTACTCTACATTTCT
	CcxEi10_856	*Caretta caretta* x *Eretmochelys imbricata*	856CcxEi Hybrids	SAMN39212792	AACAGCCTTAGTCCCCGARCTCAAAGGAGCACTGACTGCTATTTTTGTGTGTCCCCAGAGGTGAAT CTAATCCCAAGGAGGGCAAAGATGGAGGAGGAACYCTGCCAGGCAGAGCAGGCTGMACCATCCT TAAGTTCAGTGTACTCTACATTTCT

Table S5. Haplotypic map of loci for each analysed individual. Blank squares are missing haplotyping data. Refer colour legends for haplotypes and individuals.

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