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RESEARCH ARTICLE

Integrating morphological, molecular and cytogenetic data for F2 sea turtle hybrids diagnosis revealed balanced chromosomal sets

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Abstract

Hybridization could be considered part of the evolutionary history of many species. The hybridization among sea turtle species on the Brazilian coast is atypical and occurs where nesting areas and reproductive seasons overlap. Integrated analysis of morphology and genetics is still scarce, and there is no evidence of the parental chromosome set distribution in sea turtle interspecific hybrids. In this study, chromosome markers previously established for pure sea turtle species were combined with morphological and molecular analyses aiming to recognize genetic composition and chromosome sets in possible interspecific hybrids initially identified by mixed morphology. The data showed that one hybrid could be an F2 individual among *Caretta caretta* × *Eretmochelys imbricata* × *Chelonia mydas*, and another is resulting from backcross between *C. caretta* × *Lepidochelys olivacea*. Native alleles of different parental lineages were reported in the hybrids, and, despite this, it was verified that the hybrid chromosome sets were still balanced. Thus, how sea turtle hybridism can affect genetic features in the long term is a concern, as the implications of the crossing-over in hybrid chromosomal sets and the effects on genetic function are still unpredictable.

KEYWORDS
Cheloniidae, Cryptodira, diagnostics chromosomes, endangered species, introgression

1 | INTRODUCTION

Natural hybridization is the successful interbreeding between distinct genomes (Bérubé & Palsbøll, 2018; Harrison & Larson, 2014; Rhymer & Simberloff, 1996), different species or subspecies, combining specific and previously isolated gene pools (Bérubé & Palsbøll, 2018; MacPherson et al., 2023; Piett et al., 2015). The interbreeding can lead to different genetic results, such as (1) between pure lines leading to a first-generation hybrid (F1); (2) between F1 producing a second-generation hybrid (F2) and (3) hybrids that interbreed with a pure parental lineage (Piett et al., 2015; Purves et al., 2001). Hybrids resulting from backcrosses usually have a

morphology more similar to the parental species (Piett et al., 2015; Purves et al., 2001) and could have more chances of viable offspring (Soares et al., 2018). The continuous process of hybridization can lead to introgression, i.e., when one species' alleles are incorporated into a distinct gene pool (Rhymer & Simberloff, 1996; Vilaça et al., 2012).

Considering species where hybridization is rare on a per-individual basis, for example, in vertebrates, hybrids are usually inviable or sterile, maintaining the genetic pool integrity of the pure lines (Mallet, 2005; Mayr, 1963). On the other hand, species that commonly hybridize tend to produce viable hybrids, which can reproduce and consequently reduce or even eliminate the reproductive isolation between parental species (Bérubé & Palsbøll, 2018). Moreover, genetic introgression, in some cases, can promote speciation, hybrid vigour or create permanent hybrid zones (Abbott et al., 2013; Bérubé & Palsbøll, 2018; Noronha et al., 2022; Rhymer & Simberloff, 1996). Thus, the hybridization event can be considered both a negative process, contributing to extinction episodes or a positive one, contributing to population adaptation, rescuing populations threatened by inbreeding, and preserving biodiversity (Pekkala et al., 2012; Piett et al., 2015).

All Cheloniidae species that share Brazilian beaches as nesting sites have suffered under prolonged anthropic pressure, which has caused the decline of populations (IUCN, 2021; Lara-Ruiz et al., 2006). Loggerhead turtle (*Caretta caretta*), olive ridley (*Lepidochelys olivacea*), green turtle (*Chelonia mydas*) and hawksbill turtle (*Eretmochelys imbricata*) have been reported to be involved in hybridization cases (Arantes, Ferreira, et al., 2020; Arantes, Vilaça, et al., 2020; Conceição et al., 1990; Lara-Ruiz et al., 2006; Proietti et al., 2014; Soares et al., 2017, 2018; Vilaça et al., 2012, 2021, 2022; Vilaça & Santos, 2013). Hybridism cases were observed around the world among *C. caretta* × *C. mydas*, *C. mydas* × *E. imbricata*, *C. caretta* × *E. imbricata*, *L. olivacea* × *E. imbricata* and *L. olivacea* × *C. caretta* (James et al., 2004; Karl et al., 1995; Seminoff et al., 2003; Wood et al., 1983). Nevertheless, the sea turtle hybridism on the Brazilian northeast coast is atypical, reaching 42% of hybrid females in nesting sites, higher than in any other analysed population worldwide (Arantes, Vilaça, et al., 2020; Lara-Ruiz et al., 2006; Reis, Soares, & Lôbo-Hajdu, 2010; Reis, Soares, Vargas, et al., 2010; Vilaça et al., 2021).

Hybridization zones occur where the nesting areas and reproductive seasons of two or more species of sea turtles overlap (Soares et al., 2017). Studies have shown that most sea turtles' hybrids correspond to the F1 generation, whereas F2 hybrids were confirmed only in hatchlings and juveniles (Arantes, Vilaça, et al., 2020). This fact suggests that F2 hybrids may not survive to adulthood, or the hybridization events reported in Brazil are recent, around 20 years (Arantes, Vilaça, et al., 2020; Vilaça et al., 2012). On the other hand, using whole genome data analysis, Vilaça et al. (2021) assumed that hybridization events within Chelonioidea may have occurred millions of years after their initial divergence, constituting a case of speciation with gene flow in the sea turtles. In another study, Vilaça et al. (2022) developed a new tool to analyse mitochondrial DNA (mtDNA) in sea turtles and reported that most fertile hybrids have mtDNA from both parental species as a result of paternal leakage. In the same study, based on genomic data, they proposed that all adult hybrids analysed are first generation (F1) suggesting a strong hybrid breakdown in the second generation, and consequently, the sea turtle species continue to maintain distinct genetic pools and separate evolutionary trajectories (Vilaça et al., 2022).

Sea turtles exhibit significant phenotypic variability in the number and shape of carapace scutes and head scales (Mast & Carr, 1989; Ozdemir & Turkozan, 2006; Pritchard & Mortimer, 1999; Sim et al., 2014; Wyneken, 2001). However, this variability can also occur due to hybridization, making the analysis of morphological parameters one of the first steps to identify potentially hybrid sea turtles and thus screen them for further genetic analysis (Garofalo et al., 2012; Hart et al., 2019; Kelez et al., 2016; Sim et al., 2014). Studies of hybrids with molecular markers allow the analysis of hybridization events with better precision than morphology-based approaches, constituting a tool for identifying the parental lineages (Seminoff et al., 2003; Vilaça et al., 2012).

Cytogenetic analysis is a valuable tool for identifying hybrids based on karyotypes, as documented for the genus *Mazama* (Galindo et al., 2021), *Sus scrofa* (Wnuk et al., 2005), Amphibia (Haddad et al., 1994) and in different genera of fish (Káldy et al., 2020; Pomianowski & Ocalewicz, 2021). It becomes an essential tool because hybrids result from two different chromosome sets (Machado, Domit, et al., 2020; Machado, Glugoski, et al., 2020). These differences can trigger errors during meiosis, such as the incorrect pairing of parental chromosomes, chromosome segregation errors and unequal crossing-over (Dobigny et al., 2017; Galindo et al., 2021). Consequently, meiosis defects prevent genetic introgression and generate deleterious effects on individual reproductive fitness (Barbosa et al., 2017; Dobigny et al., 2017; Faria & Navarro, 2010; King, 1995).

Cheloniidae species are recognized for having a similar karyotype which probably allows the successful hybridization among different species (Bickham, 1981; Karl et al., 1995). Species-specific chromosome markers were detected in Cheloniidae species, identified by classical and molecular cytogenetics (Machado, Domit, et al., 2020; Machado, Glugoski, et al., 2020). Since these chromosome markers were detected in sea turtle pure lines, they can help to detect the parental chromosomes in the hybrids' karyotypes (Machado, Domit, et al., 2020). Here, an integrated analysis of morphological, molecular and cytogenetics markers was applied to evaluate two new cases of putative hybridization in sea turtles, aiming to investigate the chromosomal sets in living hybrids.

2 | MATERIALS AND METHODS

2.1 | Sampling

Biological samples were obtained from 51 individuals of four sea turtle species (*C. mydas*, $N = 27$: 1 female and 26 juveniles—that is, sex unidentified; *C. caretta*, $N = 12$: 2 males, 5 females and 5

juveniles; *E. imbricata*, *N* = 6: 2 females and 4 juveniles and *L. olivacea*, *N* = 6: 1 male and 5 juveniles) of Cheloniidae that nidificate on the Brazilian coast (Table S1). Besides these, two captive juveniles initially identified as *C. mydas* and *C. caretta* were recognized as probable hybrids by a morphological analysis using characters that are commonly used for sea turtle diagnosis (Wyneken, 2001). They were submitted to morphological, molecular and cytogenetic analysis for hybrids determination. After confirmation of hybridism, the specimens were named *hybrids A* and *B* (Table S1). *Hybrid A* was maintained in captivity condition since a hatchling in the Projeto Tamar Foundation Visitor Center in Praia do Forte, Mata de São João, Bahia, Brazil. *Hybrid B* was placed in rehabilitation after being found stranded on the beach of Pontal do Sul, Paraná, Brazil. After recuperation, *hybrid B* was reintroduced to wildlife. Specimens were collected with the authorization of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), System of Authorization and Information about Biodiversity (SISBIO-Licence Ids 52218-8; 43433-2/3). All experimental procedures were authorized and performed following the Ethical Committee on Animal Use of the Universidade Estadual de Ponta Grossa (Protocol: 7200/2016).

2.2 | Morphological analyses

The morphological characteristics of the individuals were analysed based on the anatomical descriptions and diagnostic features of each species (Pritchard & Mortimer, 1999; Wyneken, 2001). First, the number of prefrontal and postorbital scales was counted, followed by the count of vertebral, lateral, nuchal, marginal and supracaudal carapace scutes. Then, some secondary characteristics were evaluated, such as the general shape of the head and carapace of the individuals, patterns of scutes on the plastron and the number of claws in flippers (Garofalo et al., 2012; Pritchard & Mortimer, 1999; Sim et al., 2014; Wyneken, 2001).

2.3 | Obtaining the DNA sequences

Tissue samples for DNA extraction were collected from *hybrids A*, *B* and *C. caretta*, *L. olivacea*, *C. mydas* and *E. imbricata* sea turtles' flippers. Genomic DNA was extracted using ReliaPrep™ gDNA Tissue Miniprep System (Promega), following the manufacturer's instructions. To recognize species-specific alleles in *hybrids A* and *B*, and identify parental species, the following markers were used: mitochondrial DNA sequences (mtDNA) *12S* (Kocher et al., 1989) and *16S* (Romano & Palumbi, 1997) and three nuclear DNA (nucDNA) markers: one exon of brain-derived neurotrophic factor—*BDNF* (Noonan & Chippindale, 2006), one exon of oocyte maturation factor—*Cmos* (Le et al., 2006), and one intron of RNA fingerprint protein 35 gene—*R35* (Fujita et al., 2004). Besides that, the 5S ribosomal DNA (rDNA) sequence was obtained for chromosome experiments using the specific primers Fw 5′-GCCACACC ACCCTGAACAC-3′ and Rv 5′-GCCTACGACACCTGGTATTC-3′ (Suárez et al., 2017).

All sequences were amplified via Polymerase Chain Reaction (PCR), containing 40 ng genomic DNA, 0.2 μM forward and reverse primers, 0.16 mM dNTPs, 1 U *Taq* DNA Polymerase (Invitrogen), and 1.5 mM $MgCl_2$ in 1× reaction buffer (200 mM Tris, pH 8.4, 500 mM KCl). The PCR conditions were as follows: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at variable annealing temperature, 2 min at 72°C, and finally, 7 min at 72°C. Amplicons were purified using the GFXTM PCR DNA and Gel DNA Purification (Ludwig Biotecnologia) and sequenced on ABI-PRISM Genetic Analyser equipment (Applied Biosystems). Then the nucleotide sequences were analysed and edited by GENEIOUS v 7.1.9 (Kearse et al., 2012), submitted to an identity analysis on BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST) (Altschul et al., 1990) and RFAM (Kalvari et al., 2020).

2.4 | Molecular analyses of hybrids

Sequences of mtDNA and nucDNA were checked and corrected using the software Geneious (Kearse et al., 2012), and heterozygous sites in nucDNA sequences were identified. mtDNA and nucDNA sequences from pure lines of *C. mydas*, *C. caretta*, *E. imbricata* and *L. olivacea* (Naro-Maciel et al., 2008; Vilaça et al., 2012) were mined from the GenBank database (Table S2) and aligned with the hybrid's sequences using the CLUSTALW algorithm implemented in GENEIOUS software. For the nucDNA sequences, the phased haplotypes were determined using the PHASE tool (Stephens & Donnelly, 2003) available in DNASP v5 (Librado & Rozas, 2009). The PHASE runs were conducted under default parameters (100 iterations with 100 burn-in and a thinning interval of 10) and a minimum posterior probability of the haplotypes as 0.9. The haplotype (h) and nucleotide (π) diversities and the numbers of polymorphic (S) and parsimony informative sites for each gene were calculated in DNASP v5. Haplotype networks were generated using concatenated mtDNA sequences and each nucDNA marker, through the Minimum Spanning Network criterion (Bandelt et al., 1999) in POPART 1.7 (Leigh & Bryant, 2015).

2.5 | Conventional and molecular cytogenetics

Chromosomal preparations were obtained by temporary culture of lymphocytes using peripheral blood (Rodríguez et al., 2003). Slides containing chromosomal preparations were submitted to conventional 5% Giemsa staining (with pH 6.8 phosphate buffer), C-banding (Sumner, 1972), G-banding (Seabright, 1971) and fluorescence in situ hybridization (FISH) assays.

Partial 5S (obtained in this study) and 18S rDNA probes (described by Machado, Glugoski, et al., 2020), obtained from a pure line of *C. mydas*, were labelled by PCR using digoxigenin-11-dUTP and biotin-16-dUTP (Jena Bioscience), respectively. Microsatellite probes, previously described by Machado, Domit, et al. (2020) on chromosomes of pure sea turtle species, were hybridized on the

hybrids' metaphases. $(CA)_{15}$, $(GA)_{15}$ and $(GATA)_8$ microsatellites were directly labelled with Cy3 fluorochrome (Sigma-Aldrich) at the 5′ end during DNA synthesis.

FISH was performed according to Pinkel et al. (1986) and was conducted under high-stringency conditions. The hybridization mixture (2.5 ng/μL probe, 50% formamide, 2× saline-sodium citrate buffer and 10% dextran sulfate) was applied on the slides, which were then incubated for 16 h at 37°C. Anti-digoxigenin rhodamine (Roche Applied Science) and Streptavidin Alexa Fluor 488 (Molecular Probes) were used for 5S and 18S rDNA probes detection, respectively. Chromosomes were counterstained with 0.2 μg/mL 4′,6-diamidino-2-phenylindole—DAPI (Sigma-Aldrich) in the Vectashield mounting medium (Vector). The images were captured in a DFC3000 G CCD camera coupled with an epifluorescence microscope Leica DM 2000 (Leica). Twenty metaphases were analysed per sample for rDNAs and microsatellite signals detection.

Chromosomes were classified as bi-armed (meta/submetacentric and subtelocentric) or one-armed (acrocentric), and as macrochromosomes or microchromosomes (mc), according to Bickham et al. (1980). Then, the chromosomes from sea turtle *hybrids A* and *B* were arranged by decreasing size and centromere position, using as reference the pure species karyotypes (Machado, Domit, et al., 2020; Machado, Glugoski, et al., 2020). Representative idiograms of the four pure species were designed, illustrating the data obtained in the present study (5S rDNA) and those described by Machado, Glugoski, et al. (2020); Machado, Domit, et al. (2020). All microsatellite sites were positioned on the hybrid karyotypes based on their locations in pure lineages (Machado, Domit, et al., 2020).

3 | RESULTS

3.1 | Morphological analyses

All 51 individuals sampled of the *C. caretta*, *L. olivacea*, *C. mydas* and *E. imbricata* demonstrated diagnostic morphological features compatible with each species. Besides these, two captive juveniles showed mixed morphological features among species, indicating hybrid formation (Table 1). *Hybrid A*, which presents general morphological characteristics of *C. mydas*, also has similar traits to three other Cheloniidae species (Table 1; Figure 1). Three postocular scales were observed, such as present in *C. caretta*, *E. imbricata* and *L. olivacea* species (Table 1; Figure 1a). Besides that, *hybrid A* showed six vertebral scutes (Table 1; Figures 1b and 2), whereas lateral scutes were asymmetric, showing six to left side and four to right size (Table 1; Figure 1b,c). Analysis of secondary morphological traits revealed strongly serrated marginal scutes (Figures 1b, in detail, and 2), similar to *C. caretta* and *E. imbricata*, and lightly imbricated carapace scutes as in *E. imbricata* (Figures 1c, in detail, and 2).

Hybrid B presents the general morphology of a *C. caretta* with characteristics similar to those observed in *L. olivacea* (Table 1; Figure 1d). Six vertebral scutes were noted in *hybrid B* (Table 1; Figure 1e). A more detailed analysis of the *hybrid B* morphology revealed some traits comparable to olive ridley, such as a generally rounded carapace shape and marginal scutes without *C. caretta* characteristic serration (Figures 1e and 2). Also, *hybrid B* showed a single claw on each flipper (Figure 1e, in detail), and a 4th inframarginal scute on the left side of the plastron (Figure 1f, in detail).

3.2 | Nucleotide sequence analyses

Independent matrices were obtained for each amplified sequence from hybrids after being aligned with the pure haplotypes and included 394 bp for *12S*, 565 bp for *16S*, 559 bp for *BDNF*, 601 bp for *Cmos* and 439 bp for *R35* (GenBank accession IDs OR529472–OR529475 and OR533309–OR533314), resulting in a total of 2558 bp analysed. The mtDNA sequences presented 87 parsimony informative sites (Table S3), and the haplotype network showed species-specific haplotypes (Figure 3a). For nucDNA sequences, 40 parsimony informative sites were found (Table S3). *BDNF* gene presented seven polymorphic sites and the haplotypes allow the identification of the species *C. mydas* and *L. olivacea*, whereas *C. caretta* and *E. imbricata* share a unique haplotype (Figure 3b). *Cmos* gene presented 20 informative sites (Table S3), and the four species showed exclusive haplotypes (Figure 3c). *R35* sequences had 13 informative sites (Table S3) and showed exclusive haplotypes for each species (Figure 3d).

Hybrids A and *B* showed haplotypes for concatenated mtDNAs sequences (*12S* and *16S*) related to *C. caretta* (Table 1; Figure 3a). *Hybrid B* has a new mtDNA haplotype possessing four mutations to closer *C. caretta* haplotype (Figure 3). Regarding the nucDNA sequences, *Hybrid A* presented a new haplotype similar to *C. mydas* (with one mutation), and one identical to the haplotype shared between *C. caretta* and *E. imbricata* for the *BDNF* sequence (Table 1; Figure 3b). In the *Cmos* and *R35* sequences, a haplotype identical to *C. mydas* and another identical to *E. imbricata* were found (Table 1; Figure 3c,d). *Hybrid B* showed the haplotypes identical to that shared between *C. caretta* and *E. imbricata* for the *BDNF* sequence (Table 1; Figure 3b), besides *C. caretta* haplotypes for *Cmos* and *R35* sequences (Table 1; Figure 3c,d). The 5S rDNA partial sequence obtained from *C. mydas*, *C. caretta*, *E. imbricata* and *L. olivacea* (GenBank accession IDs OP661360.1–OP661363.1) showed high identity with 5S rDNA from *Bufos americanus* in GenBank and Rfam databases (Table S4).

3.3 | Cytogenetic analyses

Chelonia mydas, *C. caretta*, *E. imbricata* and *L. olivacea* showed diploid chromosome number (2n) = 56 (Figure 4) and homeologies in longitudinal G bands (Figure S1), as reported by Machado, Glugoski, et al. (2020); Machado, Domit, et al. (2020). Among sea turtle karyotypes, distinct chromosome morphologies were found by Machado, Glugoski, et al. (2020) in chromosome pairs 4, 5, 7 and 12, which were evaluated regarding the morphology and G-band positions (Figures 4 and S1). The main differences among the

TABLE 1 Morphological, mtDNA, nucDNA and cytogenetic features for *Caretta mydas*, *C. caretta*, *Eretmochelys imbricata*, *Lepidochelys olivacea*, and *hybrids A* and *B*.

		Caretta mydas		*Caretta caretta*		*Eretmochelys imbricata*		*Lepidochelys olivacea*		*Hybrid A*		*Hybrid B*	
		L	R	L	R	L	R	L	R	L	R	L	R
Head scales	Prefrontal	1	1	2	2	2	2	2	2	1	1	2[a]	2[a]
	Postocular	4	4	3	3	3	3	3	3	3[a]	3[a]	3[a]	3[a]
Carap. scutes	Lateral	4	4	5	5	4	4	≥6	≥6	6	4[b]	5	5
	Vertebral	5		5		5		≥6		6		6	
	Marginal	11	11	12	12	11	11	11	11	11[c]	11[c]	12	12
	Nuchal	1		1		1		1		1		1	
	Supracaudal	2		2		2		2		2		2	
mt	12S and 16S	CM		CC		EI		LO		CC		CC	
nuc	BDNF	CM		CC		EI		LO		CM/CC,EI[d]		CC	
	Cmos	CM		CC		EI		LO		CM/EI		CC	
	R35	CM		CC		EI		LO		CM/EI		CC	
Kar.	Pair 4	Bi-a		Bi-a		One-a		One-a		Bi-a[e]/One-a		Bi-a/One-a	
	Pair 5	Bi-a		Bi-a		One-a		One-a		Bi-a[e]/One-a		Bi-a/One-a	
	Pair 7	One-a		Bi-a		One-a		One-a		One-a[c]		Bi-a/One-a	
	Pair 12	Bi-a		Bi-a		One-a		Bi-a		Bi-a[f]/One-a		Bi-a[f]	
	NF	76		78		70		72		73		75	
CA	Pairs	4, 16		13, 17		4, 9, 13, 16		4, 14		4[c], 13[d], 16, 17		4[g], 13, 14[g], 16[g], 17	
GA	Pairs	14		13, 14		24, 25		14		13, 14[f], 24		13, 14[f]	
GATA	Pairs	13, 15, 16, 22		13, 16		9, 16, 18, 19, 24		13, 15		13[f], 15, 16[h], 18, 19, 22		13[f], 15, 16	

Note: Features representative of each lineage were highlighted in colours: *C. mydas* (red), *C. caretta* (yellow), *E. imbricata* (pink) and *L. olivacea* (blue). In hybrids features, just *C. mydas*, *C. caretta* and *E. imbricata* were considered in *hybrid A* origin and *C. caretta* and *L. olivacea* for *hybrid B* origin. When was not determined the origin, symbols were used to share features between sea turtles lineages.

Abbreviations: Carap., carapace; Kar., karyotype; L, left side; mt, mtDNA; nuc, nucDNA; R, right side.

[a]Feature shared between *C. caretta*, *E. imbricata* and *L. olivacea*.

[b]Feature shared between *C. mydas* and *E. imbricata*.

[c]Feature shared between *C. mydas*, *E. imbricata* and *L. olivacea*.

[d]Feature shared between *C. caretta* and *E. imbricata*.

[e]Feature shared between *C. mydas* and *C. caretta*.

[f]Feature shared between *C. mydas*, *C. caretta* and *L. olivacea*.

[g]Variable sites mapped in *C. caretta* individuals.

[h]Feature shared between *C. mydas*, *C. caretta* and *E. imbricata*.

karyotypes are summarized as follows: (1) chromosome pair 4, bi-armed in *C. mydas* and *C. caretta* and one-armed in *E. imbricata* and *L. olivacea*; (2) chromosome pair 5, bi-armed in *C. mydas* and *C. caretta* and one-armed in *E. imbricata* and *L. olivacea*; (3) chromosome pair 7, bi-armed in *C. caretta* and one-armed in *C. mydas*, *E. imbricata* and *L. olivacea*; (4) chromosome pair 12, bi-armed in *C. mydas*, *C. caretta* and *L. olivacea* and one-armed in *E. imbricata* (Table 1; Figure S1). Additionally, the karyotypes presented differences in repetitive DNA site distribution and number among chromosomes of the four sea turtles (Figure S2).

For the first time, 5S rDNA sites were in situ located on the sea turtle chromosomes. 5S rDNA probe markers were detected interstitially on the short arm of the second metacentric pair in *C. mydas*, *C. caretta*, *E. imbricata* and *L. olivacea* karyotypes (Figure 4). Moreover, an additional 5S rDNA site was co-located with 18S rDNA on the fourteenth mc on the *C. mydas* karyotype (Figure 4).

Hybrid A presented 2n = 56 arranged in 17 bi-armed and 39 one-armed chromosomes, and the fundamental number (FN) = 73 (Figure 5a). Chromosomes 4, 5 and 12 were heteromorphic, i.e., subtelocentric/acrocentric for 4 and 5, and metacentric/acrocentric for 12 (Table 1; Figure 5a,b). The 5S rDNA was located in the short arm of the metacentric 2, 18S rDNA markers were detected in mc pair 14, and just one chromosome member of mc pair 14 showed co-located 5S/18S rDNA sites (Figure 5c). $(CA)_{15}$ sites were located in the short arms of the subtelocentric/acrocentric pair 4 and in four mc, such as one chromosome member of pair 13, in pair 16 and just

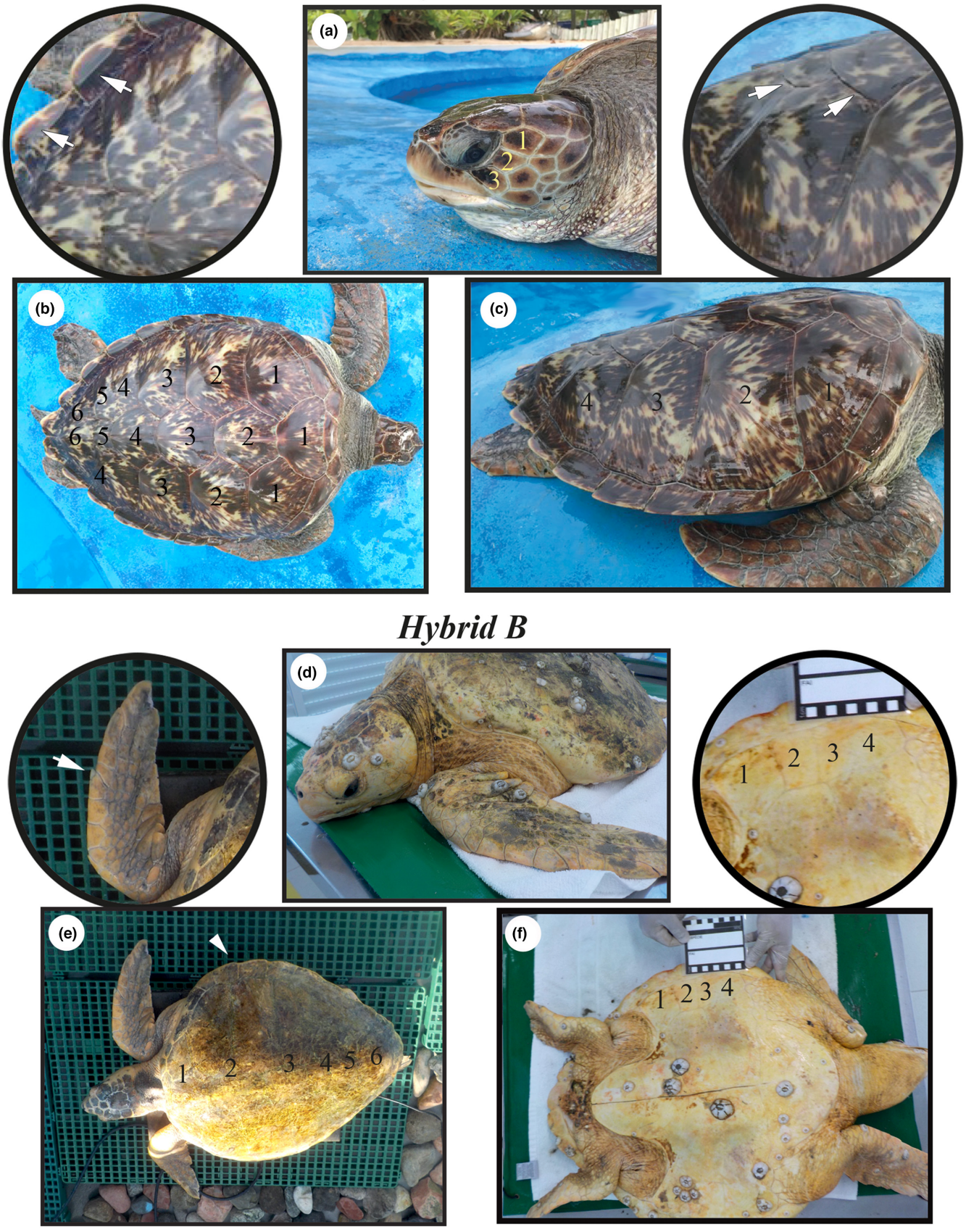
Hybrid A
(a)
1
2
3
(b)
5 4 3 2 1
6
6 5 4 3 2 1
4 3 2 1
(c)
4 3 2 1
Hybrid B
(d)
1 2 3 4
(e)
1 2 3 4 5 6
(f)
1 2 3 4

FIGURE 1 Photographs of *hybrids A* and *B* showing morphological traits considered altered or atypical of *Caretta mydas* and *C. caretta*. *Hybrid A*: (a) number of postocular scales; (b) number and morphology of the scutes, in detail, strong serrilated marginal carapace scutes and (c) number of lateral scutes in the right side, in detail, lightly imbricated vertebral carapace scutes. *Hybrid B*: (d) a general mixed morphology between *C. caretta* and *Lepidochelys olivacea*; (e) number of vertebral scutes and an overall shape of the carapace, in detail, a single claw on the right flipper and (f) ventral view of the specimen, in detail, presence of an extra 4th inframarginal scute on the plastron.

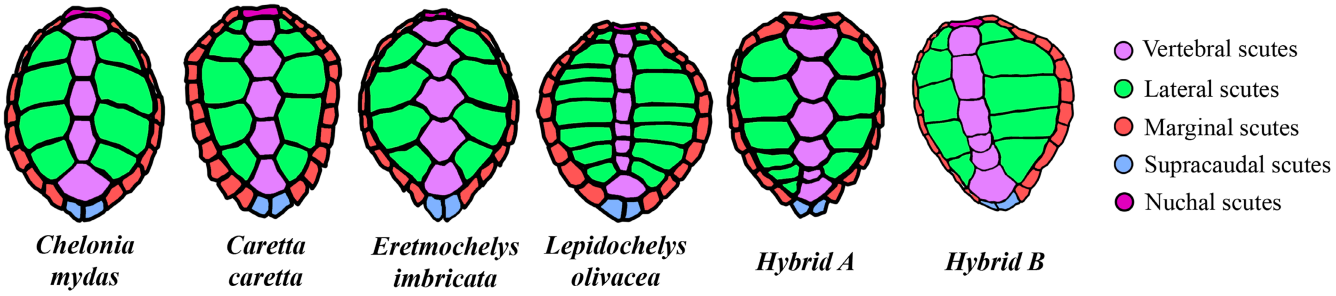

FIGURE 2 Comparison among carapace scute patterns of *Caretta mydas*, *C. caretta*, *Eretmochelys imbricata*, *Lepidochelys olivacea*, *hybrid A* and *hybrid B*. Vertebral, lateral, marginal, supracaudal and nuchal scutes are displayed in different colours.

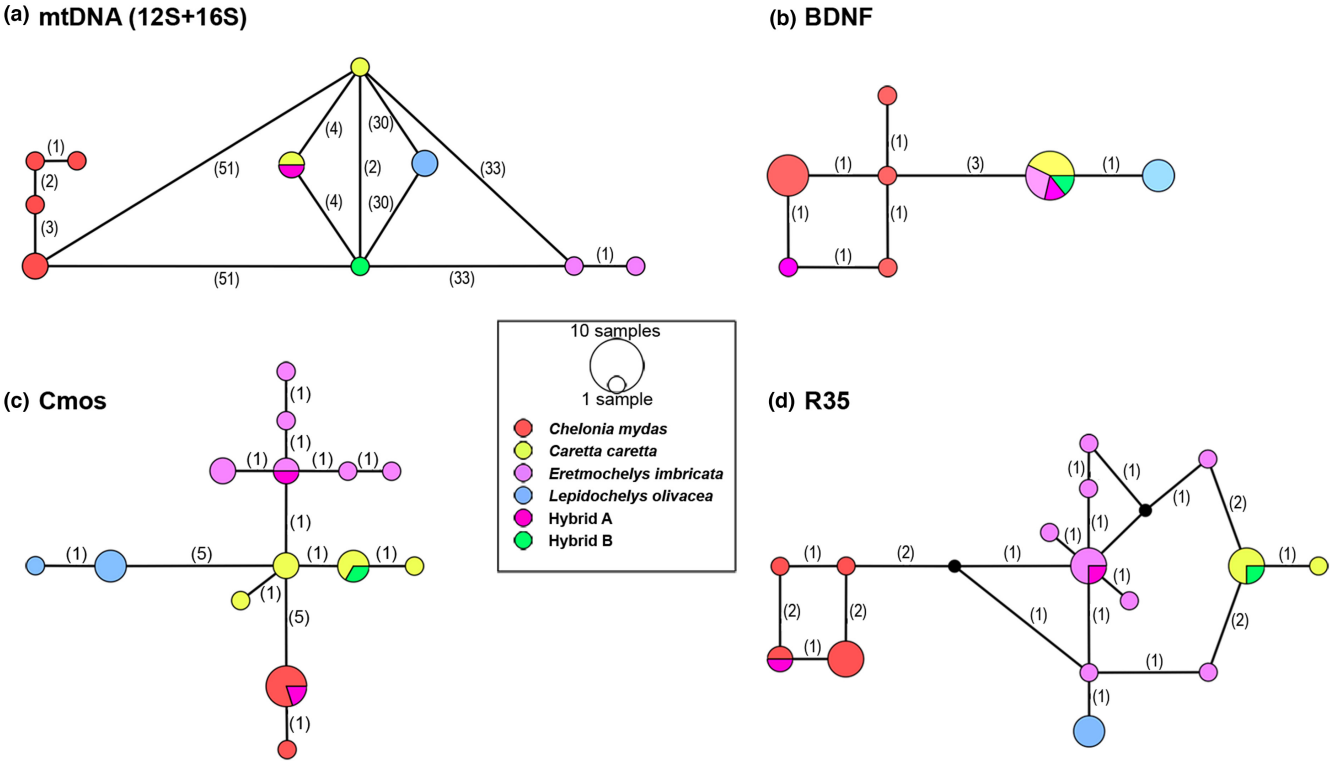

FIGURE 3 Haplotype network based on fragments of the concanated *12S* and *16S* mtDNAs (a), and on fragments of the nucDNAs (b) *BDNF*, (c) *Cmos* and (d) *R35* for *hybrids A* and *B*, and pure sea turtle's species. The numbers represent mutational differences between each haplotype.

in one member of 17 (Table 1; Figure 5d). $(GA)_{15}$ signals were detected in four mc, one member of pair 13, pair 14 and one member of 24 (Table 1; Figure 5e). $(GATA)_8$ motifs were located in eight mc sites: pairs 13 and 16, and one chromosome member of the mc 15, 18, 19 and 22 (Table 1; Figure 5f).

Hybrid B showed 2n = 56 arranged in 19 bi-armed, 37 one-armed and FN = 75 (Figure 6a). Chromosomes in the karyotypic positions 4, 5 and 7 were heteromorphic, i.e., subtelocentric/acrocentric (Table 1; Figure 6a,b). 5S rDNA sequences were in situ located in the short arm of the metacentric pair 2, whereas the 18S rDNA sites were located in mc pair 14 (Figure 6c). $(CA)_{15}$ motifs were located on the acrocentric 4, and in mc pairs 13, 14, 16 and 17 (Table 1; Figure 6d). $(GA)_{15}$ microsatellites were detected in mc pairs 13 and 14 (Table 1; Figure 6e). $(GATA)_8$ signals also were located in four mc: pair 13 and just in one mc for positions 15 and 16 (Table 1; Figure 6f).

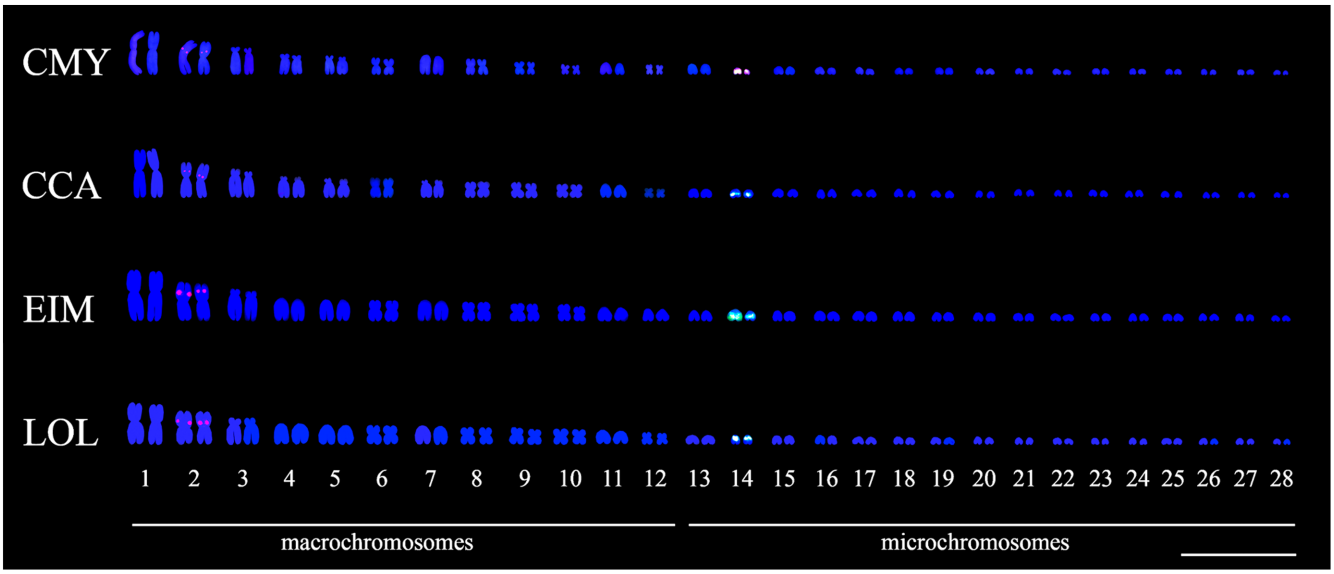

FIGURE 4 Karyotypes of *Caretta mydas* (CMY), *C. caretta* (CCA), *Eretmochelys imbricata* (EIM) and *Lepidochelys olivacea* (LOL) pure lineages subjected to FISH using 5S (red signals) and 18S rDNAs (green signals) probes. In addition, note the distinct morphology of chromosome pairs: chromosome 4 (subtelocentric in CMY and CCA, and acrocentric in EIM and LOL); chromosome 5 (subtelocentric in CMY and CCA, and acrocentric in EIM and LOL); chromosome 7 (subtelocentric in CCA and acrocentric in CMY, EIM and LOL); chromosome 12 (metacentric in CMY, CCA and LOL, and acrocentric in EIM). Metaphases (raw data) used for karyotyping are provided in Figure S3. Bar = 10 μm.

4 | DISCUSSION

Integrated data of morphological, molecular and cytogenetic features were used in our study to identify the parental species of two hybrids (*A* and *B*). The obtained data demonstrated that *hybrid A* could be an F2 among *C. caretta* × *E. imbricata* × *C. mydas*. In contrast, *hybrid B* results from backcrossing *C. caretta* × *L. olivacea*. Even though some of the morphological, molecular and chromosome features used in this study overlap in two or more pure lineages, it still is possible to detect the contribution of the parental lineages in the origin of *hybrids A* and *B*.

Morphological analysis of sea turtles is an essential filter for determining when to execute genetic analysis for hybrids recognition (Garofalo et al., 2012; Seminoff et al., 2003). Our results from *hybrids A* and *B* showed mixed morphological traits of the parental sea turtle species. The morphology analysis of *hybrid A* showed mixed diagnostic features of the species *C. caretta*, *E. imbricata* and *C. mydas*. Prefrontal head scales were equal with *C. mydas*, whereas the postocular head scales were equal with *C. caretta* and *E. imbricata*. Also, the number of carapace lateral scutes on the right side was equal to *C. mydas*, whereas, on the left side, this number is similar to *C. caretta*, *E. imbricata* and *L. olivacea*. In the same way, *hybrid A* presented numerous scales and scutes such as *C. caretta*, *E. imbricata* and *L. olivacea*, but the imbrication of the carapace scutes and the serrated marginal scutes indicates the possibility of *hybrid A* being the offspring of *C. mydas* and *E. imbricata* (Kelez et al., 2016; Pritchard & Mortimer, 1999; Seminoff et al., 2003; Wyneken, 2001).

Hybrid B assembles a general morphology of *C. caretta* with some features of *L. olivacea*, such as six vertebral scutes, a rounded carapace shape and marginal scoots without serration. The *hybrid B's* carapace demonstrated a non-modal scute pattern and alterations in plastron and flipper claw patterns (Pritchard & Mortimer, 1999; Wyneken, 2001). These traits are known for their natural variability at an individual level in sea turtle species (Garofalo et al., 2012; Pritchard & Mortimer, 1999; Wyneken, 2001). Still, these alterations, combined with the phenotypic similarity of *hybrid B*'s head and carapace to those of olive ridleys, can indicate a crossbreeding between *C. caretta* and *L. olivacea*. Hybridization between these two species was previously registered in Brazil, but is less commonly observed than between *C. caretta* and *E. imbricata* and could be the result of an overlap in reproductive seasons and features (Arantes, Vilaça, et al., 2020; Lara-Ruiz et al., 2006; Reis, Soares, & Lôbo-Hajdu, 2010; Soares et al., 2020).

In the hybrid specimens genotyping, mtDNA and nucDNAs should be used to show both female and male genetic contributions (Soares et al., 2017). Even so, Vilaça et al. (2022) considered that genomic data obtained from millions of ddRAD loci could be necessary for determining the hybrid parentals in sea turtles rather than applying just a few nuclear markers. Molecular analyses in *hybrid A* revealed that mtDNA (*12S* and *16S*) is from *C. caretta*, which suggests that a female of *C. caretta* is involved in this hybridism case. On the other hand, the nucDNAs haplotypes (*BDNF*, *Cmos* and *R35*) were suggestive of a *C. mydas* and *E. imbricata* hybrid, just with the *BDNF* haplotype overlapping feature with *C. caretta*. Hybrids among *C. caretta* × *E. imbricata* × *C. mydas* have been described on the Brazilian coast as a *C. caretta* × *E. imbricata* F1 crossed with a *C. mydas* (Vilaça et al., 2012). *BDNF* shares the same haplotype in *C. caretta* and *E. imbricata* (Vilaça et al., 2012), which makes it impossible to determine the exact order of the crosses. Thus, the *Hybrid A*' origin could be either a *C. caretta* × *E.*

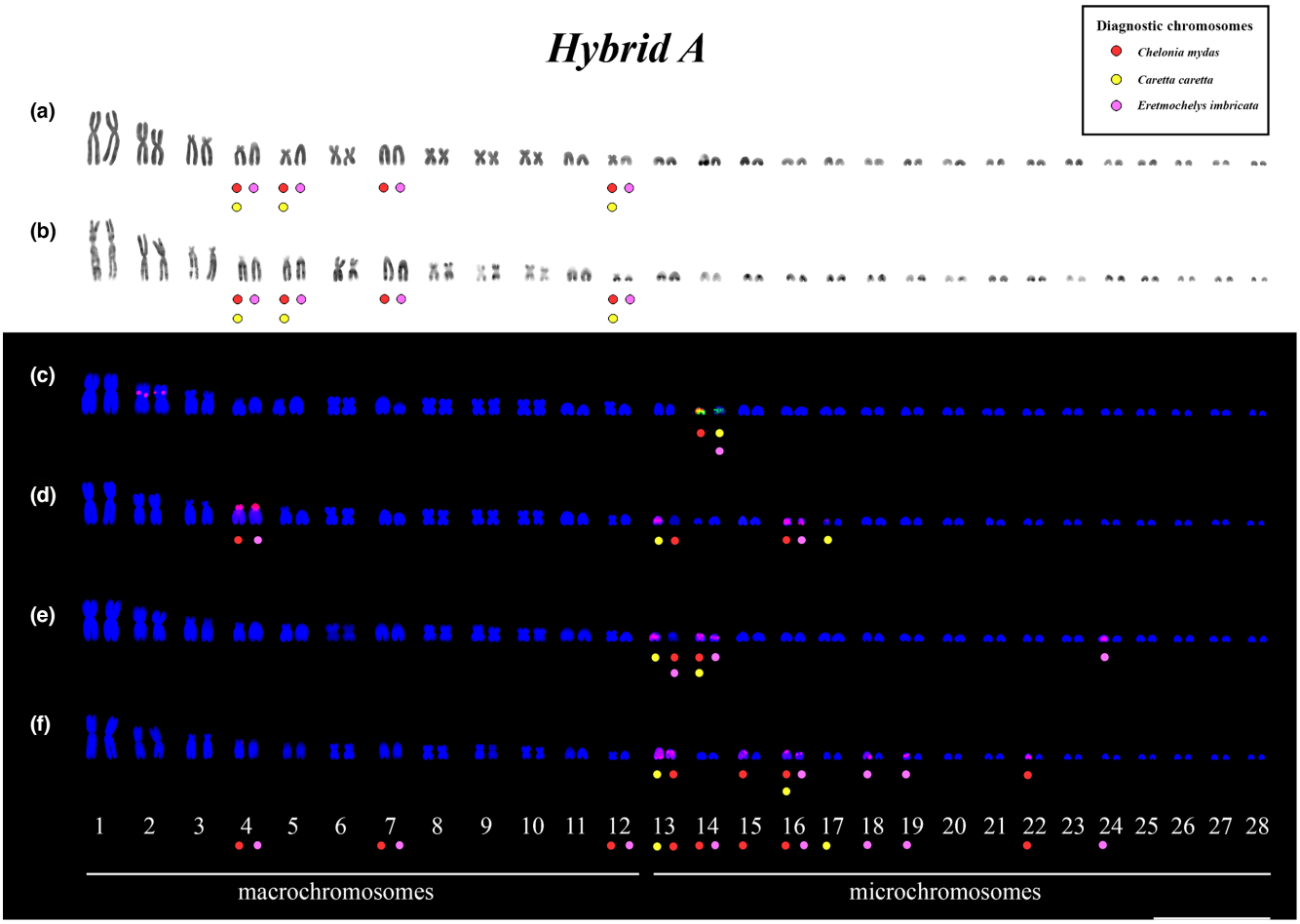

FIGURE 5 Karyotype of the *hybrid A* (identified by molecular markers as an F2 among *Caretta caretta* × *Eretmochelys imbricata* × *C. mydas*). In (a) Giemsa staining, (b) G-banding and (c–f) in situ location of chromosome markers. In (c) 5S (red signals) and 18S rDNAs (green signals), (d) $(CA)_{15}$, (e) $(GA)_{15}$ and (f) $(GATA)_8$ microsatellite probes (red signals). Based on the identification of the molecular markers, circles of different colours representing the diagnostic chromosomes from parental lineages were highlighted to represent the homeologous chromosomes 'pairs' in *hybrid A*. Metaphases (raw data) used for karyotyping are provided in Figure S4. Bar = 10 μm.

imbricata or *C. caretta* × *C. mydas* F1 that crossed a pure lineage of *C. mydas* or *E. imbricata*. On the other hand, a bias in the mtDNA analyses in the *hybrid A* could mean that this specimen would be an F1 between *C. mydas* and *E. imbricata*. Using a new approach to determine the heritage of mtDNA in sea turtles, Vilaça et al. (2022) found paternal leakage in F1 hybrids and different proportions of mitochondria from maternal and paternal species. However, if *hybrid A* is a *C. mydas* × *E. imbricata* F1, no one *C. caretta* mtDNA would be expected. Most sea turtle hybrids appear to have 50% of the alleles of each parental species, therefore being considered F1, but backcrosses with both parental species were also detected (Arantes, Vilaça, et al., 2020; Vilaça et al., 2012). *Hybrid B* presented mtDNAs of *C. caretta*, which indicates that a female *C. caretta* was involved in this case. Except for the shared haplotype of *BDNF*, the other nucDNAs haplotypes represent alleles of *C. caretta*, indicating a pure loggerhead lineage. Nevertheless, the morphology and cytogenetic analyses undoubtedly showed *L. olivacea* features in *hybrid B*. Since no alleles of *L. olivacea* were detected in the nucDNA markers, *hybrid B* is probably a *C. caretta* × *L. olivacea* hybrid backcrossed to *C. caretta*. Besides that, the data obtained here for hybrids karyotypes showed that minor differences in chromosome morphology and in situ location of cytogenetic markers helped identify the parental species involved in *hybrids A* and *B* cases.

Cytogenetically, *hybrid A* showed a karyotype with a mixed combination for diagnostic macrochromosomes, i.e., chromosomes with different morphologies at karyotype positions 4, 5, 7 and 12. Chromosome 'pairs' 4, 5 and 12 in this specimen showed a bi-armed/one-armed homeology elements. *Chelonia mydas* and *C. caretta* shared bi-armed chromosomes, whereas *E. imbricata* has one-armed chromosome in pairs 4, 5 and 12. Chromosome 7 bi-armed is diagnostic for *C. caretta* (Machado, Domit, et al., 2020; Machado, Glugoski, et al., 2020). Since *hybrid A* has a one-armed chromosome pair 7, these were a heritage from *C. mydas* and *E. imbricata*. Besides the macrochromosomes morphology, species-specific repetitive DNA sites were identified among the karyotypes of sea turtle species (Machado, Domit, et al., 2020; Machado, Glugoski, et al., 2020). Although the 18S rDNA has a chromosomal site shared among the four

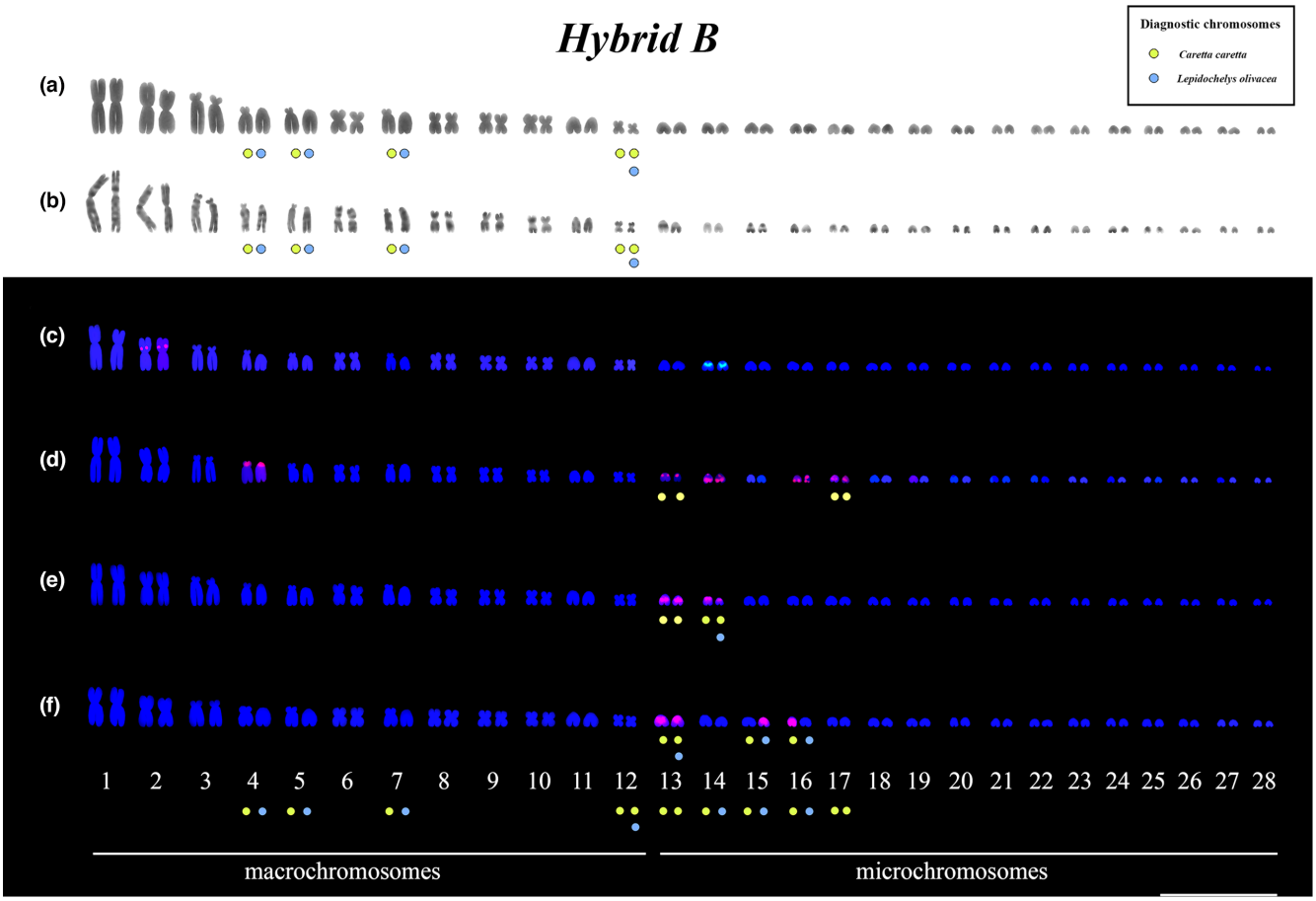

FIGURE 6 Karyotype of the *hybrid B* (identified by molecular markers as a *Caretta caretta* × *Lepidochelys olivacea* backcrossed with *C. caretta*). In (a) Giemsa staining, (b) G-banding and (c–f) in situ localization of chromosome markers. In (c) 5S (red signals) and 18S rDNAs (green signals), (d) $(CA)_{15}$, (e) $(GA)_{15}$ and (f) $(GATA)_8$ microsatellite probes (red signals). Based on the identification of the molecular markers, circles of different colours representing the diagnostic chromosomes from parental lineages were highlighted to represent the homeologous chromosomes 'pairs' in *hybrid B*. Metaphases (raw data) used for karyotyping are provided in Figure S5. Bar = 10 μm.

Cheloniidae species (Machado, Glugoski, et al., 2020) and was not useful for the recognition of sea turtle hybrids, a 5S rDNA site co-located with the 18S rDNA is a species-specific marker of *C. mydas*. The identification of 18S and 5S rDNA sites co-located in *hybrid A* (chromosome mc 14) corresponds to part of its *C. mydas* origin. In the sea turtle karyotypes (pure lineages), microsatellite loci were detected as part of repetitive units of heterochromatin, mainly in mc (Machado, Domit, et al., 2020). These chromosomal markers showed differences in the number of loci and locations among *C. mydas*, *C. caretta*, *E. imbricata* and *L. olivacea* (Machado, Domit, et al., 2020) and were considered helpful for identifying parental chromosome sets in hybridism cases, despite the variability, they could be evidence of paternal and maternal origins. The arrangement of chromosome sets in hybrids of sea turtles is arduous, especially in mc, which show the same size and morphology. Despite this extra difficulty, the data revealed that *hybrid A* has the number and location of the microsatellites on mc corresponding to *C. mydas*, *C. caretta* and *E. imbricata* origin (see Table 1; Figure 5).

In *hybrid B*, chromosome 'pairs' 4 and 5 demonstrated a bi-armed and an element with one-arm, characteristic of *C. caretta* and *L. olivacea*, besides a bi-armed chromosome diagnostic to *C. caretta* (Machado, Glugoski, et al., 2020). The chromosome 'pair' 12 showed two bi-armed chromosomes that could have an origin of just *C. caretta* or from *C. caretta* and *L. olivacea*. Thus, the karyotype of *hybrid B* showed macrochromosomes combinations between *C. caretta* and *L. olivacea*. Based on the number and location of the microsatellites on mc in pure sea turtle lineages proposed by Machado, Domit, et al. (2020), it is possible to determine mc 'pair' in *hybrid B* karyotype. Considering the mc pair, the location of sites, and the number of sites, the mc pairs 13 and 17 came just from *C. caretta*. At the same time, 14, 15 and 16 have origin from *C. caretta* and *L. olivacea*. This data supports the proposition of *hybrid B* being a backcross between *C. caretta* × *Lepidochelys olivacea*.

Still, at the karyotype level, the same longitudinal band patterns were found in chromosomes of both proposed F2 hybrids compared to those detected from their pure parental species karyotypes (Machado, Glugoski, et al., 2020). Thus, either the crossing-over did not occur in these chromosomes (at least in these heteromorphic chromosome regions), or crossing-over did not change the gene synteny between the homeologous

chromosomes. Despite the method resolution, *hybrids A* and *B* were considered to show balanced maintenance of chromosomal sets, but as the individuals are juveniles, the F2 hybrids' viability cannot be assessed.

Cases of sea turtles' hybridization on the Brazilian coast coincide with a significant population decline during the 20th century, which could lead to a reduced chance of potential conspecific encounters (Vilaça et al., 2012). Genetic introgression is a concern when F2 hybrids are found and viable, and it should be deeply studied to estimate its future consequences for pure lineages. In an evolutionary context, hybridization can be neutral, disadvantageous or adaptive (Olave et al., 2018; Piett et al., 2015). Studies have shown that the sea turtle phenomenon of hybridization in Brazil could be considered neutral (Arantes, Ferreira, et al., 2020; Arantes, Vilaça, et al., 2020; Lara-Ruiz et al., 2006; Proietti et al., 2014; Reis, Soares, & Lôbo-Hajdu, 2010; Reis, Soares, Vargas, et al., 2010; Soares et al., 2017, 2018; Vilaça et al., 2012, 2021, 2022; Vilaça & Santos, 2013). The sea turtle hybridism's positive or negative consequences remain to be intensely studied (Vilaça et al., 2021), and no F2 generation was detected as reproductively viable (Arantes, Vilaça, et al., 2020; Vilaça et al., 2012), including *hybrids A* and *B* of this study (juveniles). The F2 event in sea turtles may have been overestimated in the past, but it still exists in juveniles (Arantes, Vilaça, et al., 2020; Vilaça et al., 2012). So, the viability for adulthood and the reproductive possibility in F2 sea turtles should still be investigated. No hybrids F2 adults were observed in sea turtle nesting areas supporting the proposal that genetically-related hybrid breakdown is possibly caused by cytonuclear incompatibility (Vilaça et al., 2022). Despite the chromosome sets in *hybrids A* and *B* still being balanced, it is essential to note that mixed chromosomes (alleles) from different sea turtle lineages could generate consequences to hybrids' gene function, which could lead to inviability in F2. Since the two hybrids evaluated in this study are juveniles, our data agree with Vilaça et al. (2022), i.e., genetic barriers could lead to postzygotic reproductive isolation in sea turtle hybrids.

Conservation measures and decisions for a hybrid may depend on the cause of hybridization and its characteristics and consequences (Mallet, 2005; Moritz, 1994). Genetic diversity among Cheloniidae species was estimated at 1% of the genomic fraction (Driller et al., 2021; Vilaça et al., 2021). A genomic study indicated that Cheloniidae species had long-lasting gene flow events that continued for millions of years after the initial divergence (Vilaça et al., 2021). Here, morphological alterations were identified in *hybrids A* and *B* despite their balanced chromosomal sets. It suggests that the homeologous combination could affect genic function/expression in the development and subsequent life stages. Our results demonstrated that the cytogenetic approach could be an additional tool besides genetic/genomic analysis in sea turtle hybridism evaluation, as it can provide essential information about parental chromosome sets. They, allied to survival data, could be useful in postzygotic reproductive isolation recognition on sea turtle hybridism cases.

5 | CONCLUSIONS

Here, we observed that despite *hybrids A* and *B* presented morphological characteristics of two sea turtle species, when the analysis was integrated with molecular and cytogenetic data, we showed that they correspond to the second generation of hybrids. *Hybrid A* is an F2 result of three species hybridisms (*C. caretta* × *E. imbricata* × *C. mydas*), and *hybrid B* is a backcrossed (*C. caretta* × *L. olivacea* with *C. caretta*). It is essential to report that these individuals inherited alleles from distinct parental lines, but the chromosome sets are still balanced. Despite balanced chromosome sets, we cannot postulate how hybridism can affect long-term *hybrid B* genetics or how combined interspecific chromosomes could affect survival.

AUTHOR CONTRIBUTIONS

Caroline Regina Dias Machado: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Matheus Azambuja:** Formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – original draft (equal). **Camila Domit:** Conceptualization (equal); formal analysis (equal); funding acquisition (equal); writing – original draft (equal); writing – review and editing (equal). **Gabriel Fraga da Fonseca:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Larissa Glugoski:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Camila Borges Gazolla:** Formal analysis (equal); investigation (equal); methodology (equal); visualization (equal). **Rafael Bonfim de Almeida:** Formal analysis (equal); investigation (equal); visualization (equal). **Marcela Baer Pucci:** Investigation (equal); validation (equal); visualization (equal). **Thais Torres Pires:** Formal analysis (equal); investigation (equal); writing – original draft (equal). **Viviane Nogaroto:** Formal analysis (equal); funding acquisition (equal); investigation (equal); project administration (equal); supervision (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Marcelo Ricardo Vicari:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. No competing financial interests exist.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jeb.14236.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at URL https://doi.org/10.5061/dryad.6m905qg5g.

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