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Comparative Cytogenetics of Four Sea Turtle Species (Cheloniidae): G-Banding Pattern and in situ Localization of Repetitive DNA Units

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Keywords

Chromosome differentiation · Cryptodira · Interstitial telomeric sites · Microstructural variation · rDNA

Abstract

Sea turtles are considered flagship species for marine biodiversity conservation and are considered to be at varying risk of extinction globally. Cases of hybridism have been reported in sea turtles, but chromosomal analyses are limited to classical karyotype descriptions and a few molecular cytogenetic studies. In order to compare karyotypes and understand evolutive mechanisms related to chromosome differentiation in this group, *Chelonia mydas*, *Caretta caretta*, *Eretmochelys imbricata*, and *Lepidochelys olivacea* were cytogenetically characterized in the present study. When the obtained cytogenetic data were compared with the putative ancestral Cryptodira karyotype, the studied species showed the same diploid number (2n) of 56 chromosomes, with some variations in chromosomal morphology (karyotypic formula) and minor changes in longitudinal band locations. In situ localization using a 18S ribosomal DNA probe indicated a homeologous microchromosome pair bearing a 45S ribosomal DNA locus and size heteromorphism in all 4 species. Interstitial telomeric sites were identified in a microchromosome pair in *C. mydas* and *C. caretta*. The data showed that interspecific variations occurred in chromosomal sets among the Cheloniidae species, in addition to other Cryptodira karyotypes. These variations generated lineage-specific karyotypic diversification in sea turtles, which will have considerable implications for hybrid recognition and for the study, the biology, ecology, and evolutionary history of regional and global populations. Furthermore, we demonstrated that some chromosome rearrangements occurred in sea turtle species, which is in conflict with the hypothesis of conserved karyotypes in this group.

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Introduction

Sea turtles represent a primitive and unique component of biological diversity as members of the oldest living reptile lineage (Reptilia, Anapsida, Testudines, Cryp-

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todira) and are an important part of marine ecosystems [Rees et al., 2016]. Currently, 7 species of sea turtles are recognized in the world; one from Dermochelyidae, *Dermochelys coriacea* (leatherback sea turtle), and the other 6 from Cheloniidae: *Lepidochelys olivacea* (olive ridley sea turtle), *L. kempii* (Kemp's ridley sea turtle), *Eretmochelys imbricata* (hawksbill sea turtle), *Caretta caretta* (loggerhead sea turtle), *Chelonia mydas* (green sea turtle), and *Natator depressus* (flatback sea turtle) [Pritchard, 1997].

Multiple factors threaten sea turtle conservation, including habitat degradation and fishing mortality [Hamann et al., 2010]. Population decline, which has been recorded in most sea turtle species, drives changes in genetic variability and resiliency [Rees et al., 2016]. The study of genetic variability and sea turtle evolution are crucial for monitoring population stocks and management areas and for assessing biological and behavioral patterns, in addition to addressing aspects of animal conservation [Wallace et al., 2011]. Although interspecific hybridization in chelonian species has been reported from the last 3 decades [Lara-Ruiz et al., 2006; Proietti et al., 2014; Arantes et al., 2020], the chromosomal mechanisms involved in hybrid formation in sea turtles are poorly understood.

Nevertheless, reptiles have proven to be excellent models for understanding chromosomal evolution in cytogenetic studies [Valenzuela and Adams, 2011; Deakin and Ezaz, 2019]. This is because their karyotypes are highly diverse in terms of (i) chromosome number and morphology [Olmo, 2008; Valenzuela and Adams, 2011; Montiel et al., 2016; Deakin and Ezaz, 2019]; (ii) the absence or presence of microchromosomes (mc) [Burt, 2002; Mezzasalma et al., 2016]; (iii) macrochromosome breaks and rearrangements, which result in loss of synteny, in conflict with the hypothesis that turtle chromosomes are highly conserved [Badenhorst et al., 2015; Deakin and Ezaz, 2019; Lee et al., 2019], and (iv) sex-determining mechanisms [Valenzuela and Adams, 2011; Valenzuela et al., 2014; Rovatsos et al., 2017; Lee et al., 2019] and types of sex chromosome systems [Pokorná et al., 2011; Gamble et al., 2015; Iannucci et al., 2019; Lisachov et al., 2019; Mazzoleni et al., 2019; Nielsen et al., 2019].

The use of repetitive DNAs as chromosomal markers in reptiles has not yet been thoroughly explored. Usually, studies applying repetitive DNAs in turtles involve chromosomal localization of multigene families [Badenhorst et al., 2015; Cavalcante et al., 2018, 2020a; Matsubara et al., 2019] and in situ localization of satellite DNA sequences or transposable elements, restricted to a few groups [Badenhorst et al., 2015; Boissinot et al., 2019; Cavalcante et al., 2020b]. However, chromosomal painting and BAC-FISH for detecting single-copy genes has proven useful for identifying chromosomal rearrangements in reptiles in cytogenetic studies [Young et al., 2013; Badenhorst et al., 2015; Iannucci et al., 2019; Lee et al., 2019; Lisachov et al., 2019].

The karyotype arrangement of sea turtles has been described as the putative primitive arrangement of Dermochelyidae and Cheloniidae species, which are considered ancestors within Cryptodira [Bickham and Carr, 1983]. Furthermore, an ancestral reconstruction of diploid numbers (2n) for turtle species proposed 2n = 56 chromosomes in a sister group of Cryptodira [Valenzuela and Adams, 2011]. As such, the 2n = 56 chromosome karyotype possibly gave rise to other karyotypes through chromosomal differentiation events in the Cryptodira suborder [Bickham and Carr, 1983]. In addition, previous classic cytogenetic data show a highly conserved karyotype among sea turtles [Bickham et al., 1980; Bickham and Carr, 1983; López et al., 2008; Fukuda et al., 2012, 2014].

Only noncomparative conventional cytogenetic studies have been conducted for sea turtle species, making it difficult to establish conserved/derived chromosomal regions. Here, we performed a cytogenetic study comparing *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* with the aim of identifying possible chromosomal alterations that occurred during the diversification of sea turtle lineages.

Material and Methods

Sampling

Biological samples of *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* were collected from captive and wild populations in 5 different areas of Brazil: (i) wild *C. mydas* from Cobras Island, Paraná state and captive *C. mydas* from Extremoz, Rio Grande do Norte state and Mata de São João, Bahia state (*N* = 28: 1 female and 27 juveniles); (ii) captive *C. caretta* from Florianópolis, Santa Catarina state and Mata de São João (*N* = 11: 2 males, 5 females, and 4 juveniles); (iii) wild *E. imbricata* from Tibau do Sul, Rio Grande do Norte state and Mata de São João (*N* = 6: 2 females and 4 juveniles), and (iv) captive *L. olivacea* from Extremoz and Mata de São João (*N* = 6: 1 male and 5 juveniles).

Chromosome Preparation

Peripheral blood was used to obtain chromosomal preparations by temporary culture of lymphocytes [Rodríguez et al., 2003]. To determine the 2n and karyotypic arrangement of each turtle population, slides with chromosomal preparations were subjected to conventional 5% Giemsa staining with pH 6.8 phosphate buffer and G-banding [Seabright, 1971].

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Amplification of 18S Ribosomal DNA Sequences and Preparation of Probes

The 18S ribosomal (rDNA) sequences were amplified by polymerase chain reaction (PCR) using the 4 sea turtle genomic DNAs as templates and a primer set designed for this study (18S_fw: 5′-GTACAGTGAAACTGCGAATG-3′ and 18S_rv: 5′-CCTCGTTCATGGGGAATAAT-3′). The amplification reaction mixture contained 40 ng genomic DNA, 0.2 μM forward and reverse primers, 0.16 mM dNTPs, 1U *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 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 30 cycles of 30 s at 95°C, 45 s at 56°C, and 2 min at 72°C, and finally 7 min at 72°C. The PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL, USA) and sequenced on ABI-PRISM Genetic Analyzer equipment (Applied Biosystems, Carlsbad, CA, USA). The sequences were analyzed and their identities were confirmed on the BLASTn platform [Altschul et al., 1997].

The partial 18S rDNA sequence obtained from *C. mydas* was labeled by PCR using biotin-16-dUTP (Jena Bioscience, Dortmund, Germany). Telomeric probes – general vertebrate telomeric sequences $(TTAGGG)_n$ – were obtained according to the method of Ijdo et al. [1991] and labeled with digoxigenin-11-dUTP (Jena Bioscience).

Fluorescence in situ Hybridization

Chromosome spreads were subjected to FISH according to the method of Pinkel et al. [1986] using 18S rDNA and telomeric probes. The hybridization mixture (2.5 ng/μL probe, 50% formamide, 2× saline-sodium citrate buffer, and 10% dextran sulfate) was applied to the slides, which were then incubated for 18 h at 42°C. Streptavidin Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA) and anti-digoxigenin rhodamine antibodies (Roche Applied Science, Penzberg, Germany) were used for probe detection. Chromosomes were counterstained with 0.2 μg/mL 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, San Luis, MO, USA) in VECTASHIELD mounting medium (Vector, Burlingame, CA, USA) and observed under a Zeiss Axiolab A1 epifluorescence microscope coupled with ZEN software (Carl Zeiss, Oberkochen, Germany). At least 15 metaphases per slide/specimen were captured to confirm the fluorescence signal.

Karyotype Organization

Chromosomes were classified as bi-armed or one-armed (acrocentrics) according to their arm ratio and arranged by decreasing size and centromere position, as described by Montiel et al. [2016]. Microchromosomes were very similar (practically indistinguishable) and thus were ordered by approximate size and G-banding pattern where possible. The terms "macrochromosomes" and "microchromosomes" follow Bickham et al. [1980].

Results

The 4 species of sea turtles studied presented karyotypes of 2n = 56 chromosomes, and G-banding revealed fine chromosomal homologies in longitudinal band patterns among the karyotypes (Fig. 1). In addition, variations in karyotypic formula in terms of chromosome morphology and small differences in longitudinal band size were detected between *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* (Fig. 1). The 18S rDNA partial sequences (~1,400 bp long) obtained from *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* (GenBank accessions: MT581298–MT581301, respectively) were found to be 99% similar to *Terrapene carolina triunguis* 18S ribosomal RNA (GenBank accession No. XR_003370197.1). In order to compare the karyotypes of sea turtles obtained here to those previously published for other Cryptodira species, we propose karyotype reclassification for the studied sea turtles as follows.

C. mydas presented a karyotype of 2n = 56 chromosomes arranged in 10 bi-armed chromosome pairs (1–6, 8–10, and 12) and 18 acrocentric pairs (7, 11, and 13–28; 13–28 were mc), and fundamental number (FN) = 76 (Fig. 1). The 18S rDNA sequence was located in situ interstitially in the long arm of mc pair 14 (Fig. 2). The $(TTAGGG)_n$ probe was detected in the telomeric regions of all chromosomes (Fig. 2) in addition to an interstitial telomeric site (ITS) on the long arm of mc pair 13 (Fig. 2, box a).

C. caretta showed a karyotype of 2n = 56 chromosomes arranged in 11 bi-armed chromosome pairs (1–10 and 12) and 17 acrocentric pairs (11 and 13–28; 13–28 were mc), and FN = 78 (Fig. 1). The karyotype of *C. caretta* differed from that of *C. mydas* due to a larger band in the p arm of chromosome pair 7 in the former species (Fig. 1). One interstitial 18S rDNA site was detected in the long arm of mc pair 14 (Fig. 2). The $(TTAGGG)_n$ probe was located in the telomeric regions of all chromosomes (Fig. 2), in addition to an ITS on the long arm of mc pair 13 (Fig. 2, box b).

E. imbricata presented a karyotype of 2n = 56 chromosomes arranged in 7 bi-armed chromosome pairs (1–3, 6, and 8–10) and 21 acrocentric pairs (4–5, 7, and 11–28; 13–28 were mc), and FN = 70 (Fig. 1). The karyotype of *E. imbricata* differed from that of *C. mydas* due to the smaller size of the bands in the p arms of chromosome pairs 4, 5, and 12 in the former species (Fig. 1). The $(TTAGGG)_n$ probe was detected in the telomeric regions of all chromosomes, but no ITSs were detected (Fig. 2). The 18S rDNA sequence was located in situ interstitially in the long arm of mc pair 14 (Fig. 2, box c).

L. olivacea presented a karyotype of 2n = 56 chromosomes, arranged in 8 bi-armed chromosome pairs (1–3, 6, 8–10, and 12) and 20 acrocentric pairs (4–5, 7, 11, and 13–28; 13–28 were mc), and FN = 72 (Fig. 1). The karyo-

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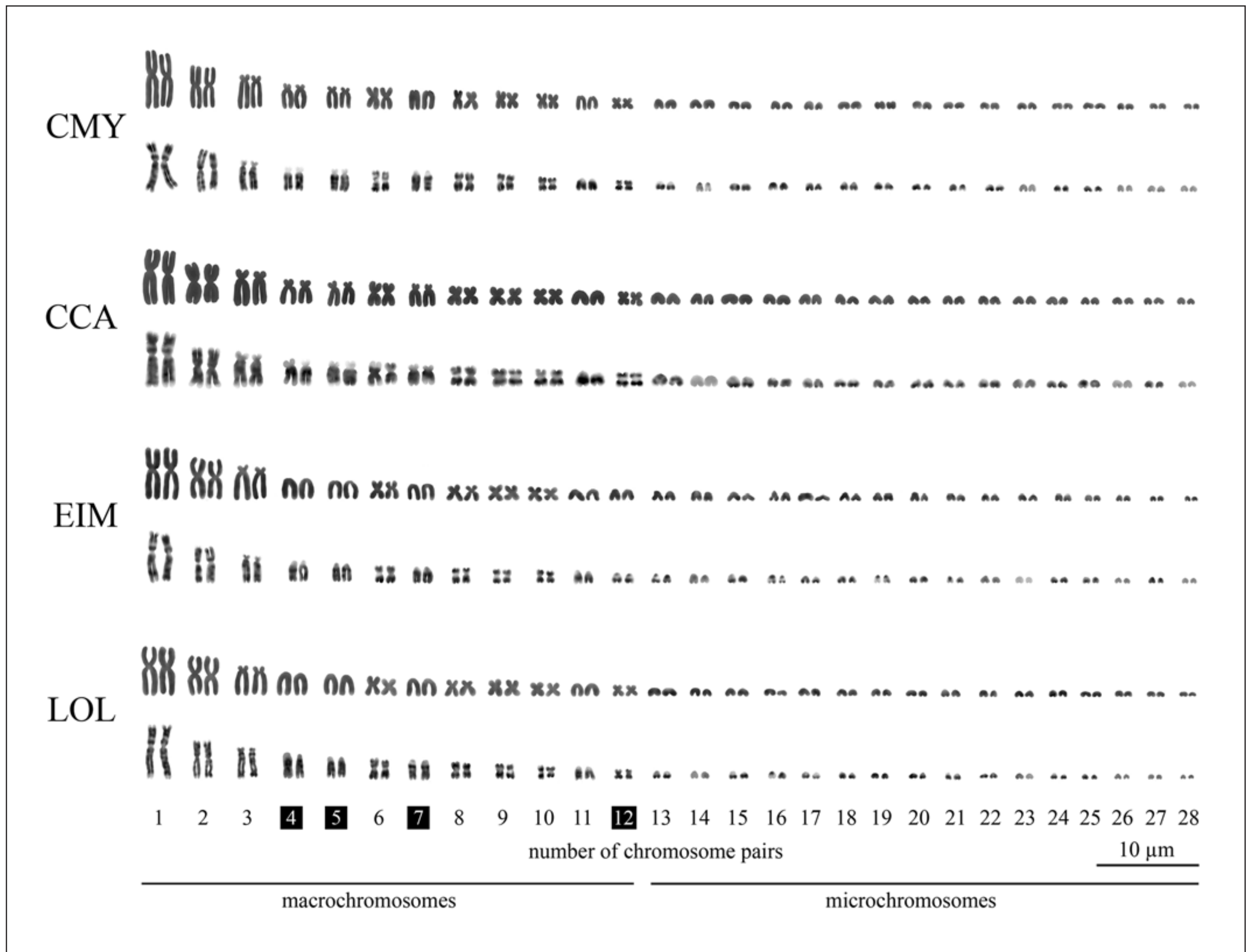

Fig. 1. Karyotypes of sea turtles subjected to Giemsa staining and G-banding. Numbers in black boxes indicate chromosome pairs showing morphological alterations. Species names are presented as 3-letter acronyms: *Chelonia mydas* (CMY), *Caretta caretta* (CCA), *Eretmochelys imbricata* (EIM), and *Lepidochelys olivacea* (LOL).

type of *L. olivacea* differed from that of *C. mydas* due to the smaller size of the bands in the p arm of chromosome pairs 4 and 5 in the former species (Fig. 1). The $(TTAGGG)_n$ probe was detected in the telomeric regions of all chromosomes, but no ITSs were detected (Fig. 2). The 18S rDNA sequence was located in situ interstitially on the long arm of mc pair 14 (Fig. 2, box d).

Discussion

In testudinates, cytogenetic data show broad variation in 2n chromosome number, which ranges from 26 to 68 [Ventura et al., 2014; Montiel et al., 2016]. In addition, cytogenetic studies in turtles have demonstrated that numerous chromosomal rearrangements, e.g., chromosome fusion, fissions, and inversions, occurred during karyotype lineage differentiation [Valenzuela et al., 2014; Montiel et al., 2016; Cavalcante et al., 2018; Lee et al., 2019; Mazzoleni et al., 2019]. Despite the chromosomal diversity among extant turtles, previous noncomparative cytogenetic studies on sea turtle species have described identical karyotypes [Bickham and Carr, 1983; López et al., 2008; Fukuda et al., 2012, 2014]. In the present study, the karyotypes of *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* were compared, and the data suggest that minor chromosomal changes occurred during the karyotypic evolution of the group.

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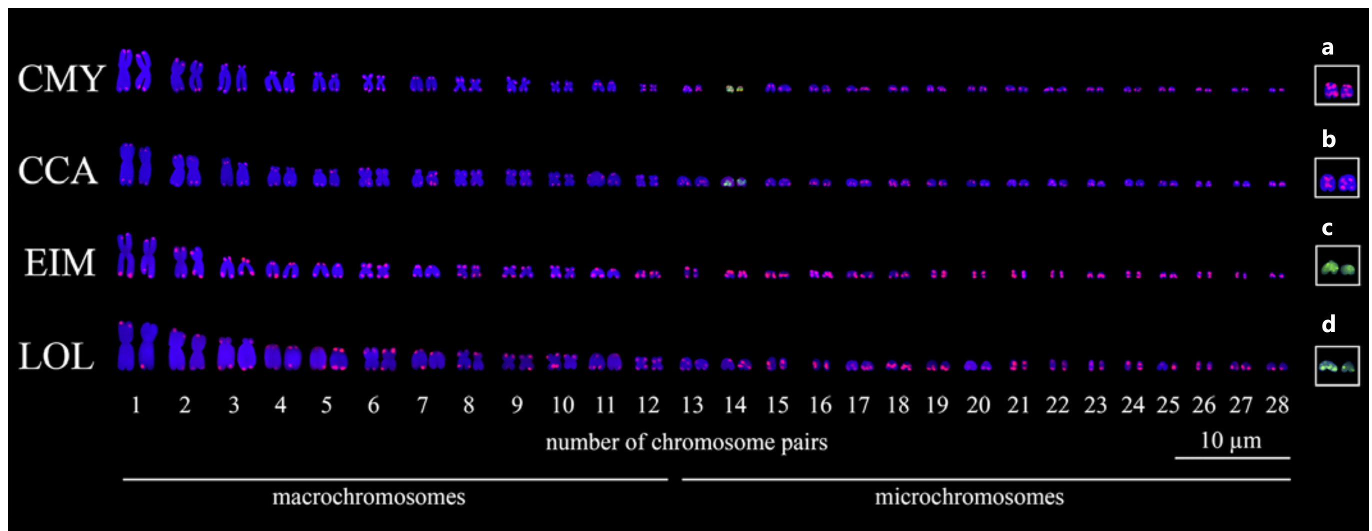

Fig. 2. Karyotypes of sea turtles subjected to FISH using probes of 18S rDNA (green signals) and $(TTAGGG)_n$ telomere repeats (red signals). Species names are presented as 3-letter acronyms: *Chelonia mydas* (CMY), *Caretta caretta* (CCA), *Eretmochelys imbricata* (EIM), and *Lepidochelys olivacea* (LOL). Boxes **a**, **b** show magnified images of microchromosome pair 13 bearing interstitial telomeric sites in CMY and CCA, respectively. Boxes **c**, **d** show magnified images of microchromosome pair 14 presenting 45S rDNA copy number variation in EIM and LOL, respectively.

The sea turtle species established as independent lineages approximately 50–10 million years ago despite overlaps among their habitats [Valenzuela and Adams, 2011; Rees et al., 2016]. The 2n = 56 chromosome karyotype was described in a sister group of Cryptodira and in *C. mydas,* which is considered to be the oldest lineage among living Cheloniidae species [for revision, see Valenzuela and Adams, 2011]. The *C. mydas* karyotype has been described as 2n = 56 chromosomes, arranged in 10 bi-armed and 18 one-armed chromosome pairs [Haiduk and Bickham, 1982], which might represent the plesiomorphic karyotypic condition of the group. Thus, we propose that minor chromosomal differentiation events might have resulted in the karyotypes observed in other Cheloniidae species, as in *C. caretta*, *E. imbricata,* and *L. olivacea* in the present study.

The data on sea turtles obtained here allowed us to detect similar karyotypes and indicate probable maintenance of synteny in major chromosome pairs, as suggested in former studies [Bickham et al., 1980; López et al., 2008; Fukuda et al., 2012, 2014]. The same G-banding chromosome pattern can be detected in some macrochromosomes of species closely related to Cheloniidae, as described by Montiel et al. [2016]. However, the present analyses also show differences in chromosomal morphology among 3 chromosomal pairs of intermediate size and chromosome pair 12 in *E. imbricata*. These differences in chromosomal morphology can be attributed to chromosomal rearrangements, such as chromosome deletion, pericentric inversion, or centromere repositioning, and show, for the first time, karyotypic diversification in the evolution of these 4 sea turtle lineages.

Pericentric inversions are thought to occur in freshwater turtle species due to 2n conservation and FN changes [Sites et al., 1979]. The occurrence of these inversions in freshwater turtle species was verified by comparing the distribution of longitudinal bands on the chromosomes [Sites et al., 1979]. However, it was not possible to detect chromosome inversions in the comparative G-banding analysis among *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* karyotypes in the present study. Centromere repositioning can also generate differences in chromosomal morphology and FN without alteration of the 2n chromosome number. The mechanism by which this occurs involves the emergence of a new centromere along the chromosome and consequent former centromere inactivation. This event profoundly affects the chromosomal architecture even if it does not change the physical order (synteny) of the chromosome markers [Montefalcone et al., 1999]. Centromere repositioning has been recognized as an important factor in karyotypic evolution, with consequences for population dynamics and speciation [Carbone et al., 2006]. However, our data do not yet allow us to state definitively that pericentric inversions or centro-

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mere repositioning resulted in the morphological diversification of chromosome pairs in sea turtles.

Repetitive DNAs are also responsible for numerous changes in chromosomal morphology as molecular mechanisms may lead to increased or decreased numbers of repeats in genomes [Smith, 1976; Charlesworth et al., 1994]. In terms of the present study, a change in the number of repeats of one DNA unit may have changed the chromosomal morphology of pairs 4, 5, and 7 among *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea*. In addition, our comparative G-banding data indicate a probable deletion in the p arm of the metacentric pair 12 of *C. mydas*, *C. caretta,* and *L. olivacea* that is not found in an acrocentric pair in *E. imbricata*.

Turtles show broad diversity in 2n chromosome number, and ITSs were found in some cytogenetic studies, suggesting that in some turtles, ITSs derive from chromosomal fusion [Montiel et al., 2016; Cavalcante et al., 2018; Srikulnath et al., 2019; Clemente et al., 2020]. Generally, ITSs in chromosomes are associated with (i) unstable chromosomal sites [Bolzán, 2017], (ii) vestigial telomeric sequences at chromosomal fusion points [Meyne et al., 1990; Glugoski et al., 2018], (iii) satellite units at heterochromatic sites [Faravelli et al., 2002], or (iv) sites of telomeric insertion during double-strand break repair with telomerase action [Azzalin et al., 2001; Ruiz-Herrera et al., 2008]. Sea turtle karyotypes retain the 2n = 56 chromosome karyotype and show no evidence of robertsonian fusion or chromosome rearrangement involving mc pair 13. Thus, the ITSs detected in both *C. mydas* and *C. caretta* mc in the present study represent a chromosomal difference compared to *E. imbricata* and *L. olivacea* that is not related to 2n chromosome number or chromosomal morphology.

The detection of nucleolus organizer regions (NORs) using silver nitrate impregnation in a previous study demonstrated that sea turtles have a single mc pair bearing a 45S rDNA locus [Bickham and Rogers, 1985]. In addition, a single chromosome pair bearing a 45S rDNA locus was found in all turtles examined in cytogenetic studies [Montiel et al., 2016]. In the present study, in situ localization of the 18S rDNA probe allowed the identification of 45S rDNA loci spread along the long arm of one probably homeologous mc pair in *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea*.

Testudinate species, which have karyotypes with a diploid number of 50–58 chromosomes, were reported to possess NORs located in an mc pair [Noleto et al., 2006; Badenhorst et al., 2015; Montiel et al., 2016]. Conversely, testudinate species and closely related groups with highly rearranged karyotypes show fusion of the mc carrying the NORs to macrochromosomes [Cavalcante et al., 2018; Matsubara et al., 2019]. The *Gallus gallus* karyotype has 45S rDNA in mc pair 16 [Auer et al., 1987; Dyomin et al., 2016], which is probably homeologous to the sea turtle mc pair visualized in the present study, supporting the theory that this is a plesiomorphic condition in turtles, crocodilians, and birds. Also, the NOR cistron visualized in the present study in the 4 sea turtle species showed extensive copy number variation among the homologues, probably as a result of unequal crossing over.

Heteromorphic sex chromosomes in trionychid species were reported to be microchromosomes; both Z and W chromosomes show 45S rDNA accumulation, and the W chromosome has a larger ribosomal unit number [Badenhorst et al., 2013; Rovatsos et al., 2017]. Even though the sea turtles showed size heteromorphism in the mc pair bearing 45S rDNA, sex determination in Cheloniidae species is environmental rather than genotypic. Environmental sex determination is considered to have evolved independently in 5 families (Chelidae, Emydidae, Geoemydidae, Kinosternidae, Trionychidae) [Valenzuela and Adams, 2011; Badenhorst et al., 2013; Rovatsos et al., 2017; Lee et al., 2019].

Variations in chromosome number and structure are factors that prevent hybrid viability [Coyne et al., 1993; Orr and Presgraves, 2000] and limit recombination rates [Rieseberg, 2001]. Interspecific hybridization in chelonian species on the coast of Brazil has been reported to be recent, and occurs mainly between hawksbill (*E. imbricata*) and loggerhead (*C. caretta*) turtles, although F2 hybrids may not survive to adulthood [Arantes et al., 2020]. Unbalanced gametes in F1 sea turtle hybrids may originate due to chromosomal differences among species. In such cases, unbalanced F1 gametes could lead to unviability in F2 turtles or subsequent generations. The chromosomal barriers to sea turtle hybridism should be better investigated using a deep cytogenetic analysis.

The present study established a karyotypic comparison among Cryptodira species and provided evidence of species-specific chromosomal differences among *C. mydas*, *C. caretta*, *E. imbricata,* and *L. olivacea* sampled on the Brazilian coast. The results are important for understanding karyotypic diversification in the evolutionary lineage of Cryptodira, especially in Cheloniidae. The data may also be useful in future studies on the karyotype structures of interspecific hybrids in the group and in studies related to sea turtle conservation.

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Statement of Ethics

All procedures with the animals were performed following the Ethical Committee on Animal Use (Protocol: 7200/2016) of the Universidade Estadual de Ponta Grossa and current Brazilian legislation.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

C.R.D.M., C.D., V.N., and M.R.V. designed the research. C.R.D.M., L.G., M.B.P., D.W.G., L.A.M., G.W.W.F.C, V.N., and M.R.V. performed the experiments. C.R.D.M., L.G., C.D., M.B.P., D.W.G., and M.R.V. analyzed the results. All authors wrote and revised the manuscript and approved the final version.

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