SUPPLEMENTAL MATERIAL

***De novo* genome assemblies of two cryptodiran (hidden-neck) turtles with ZZ/ZW and XX/XY sex chromosomes provide insights into patterns of genome reshuffling and uncover novel 3D genome folding in amniotes**

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SUPPLEMENTARY METHODS

Initial *de novo* assemblies of the *Apalone* and *Staurotypus* genomes

A *de novo* assembly for *Apalone* was constructed using a combination of paired end reads (mean insert size ~390 bp and ~402 bp). The *de novo* assembly was obtained using Meraculous v.2.2.4 (diploid_mode 1) (Chapman et al. 2011) with a k-mer size of 55. The input data consisted 738,242,104 read pairs sequenced from paired-end libraries (totaling 205.90 Gbp; 110.8 × coverage). The *de novo* assembly for *Staurotypus* was generated at a different time than for *Apalone*, from two Illumina lanes of paired-end 100 bp reads from the adult male and one adult female, and two lanes of 150 bp paired-end reads from the adult male, totaling 1,636,140,000 reads (72.1 × coverage; Table S1), which were assembled in Platanus v.1.2.4 (Kajitani et al. 2014). Reads were trimmed for quality, sequencing adapters, and mate pair adapters before assembly using Trimmomatic v.0.40 (Bolger et al. 2014).

Chicago library preparation and sequencing

Three Chicago libraries were prepared for *Apalone* and four for *Staurotypus* as described previously (Putnam et al, 2016). Briefly, for each library, ~500ng of HMW gDNA (mean fragment length = 40 kbp for *Apalone* and 50 kbp for *Staurotypus*) was reconstituted into chromatin *in vitro* and fixed with formaldehyde. Fixed chromatin was digested with DpnII, the 5' overhangs filled in with biotinylated nucleotides, and then free blunt ends were ligated. After ligation, crosslinks were reversed and the DNA purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was then sheared to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The libraries were sequenced on an Illumina HiSeq X (rapid run mode).

Dovetail Hi-C library preparation and sequencing (multiple libraries)

Three Dovetail Hi-C libraries were prepared for *Apalone* and two for *Staurotypus* in a similar manner as described previously (Lieberman-Aiden et al. 2009). Briefly, for each library, chromatin was fixed in place with formaldehyde in the nucleus and then extracted. Fixed chromatin was digested with DpnII, the 5' overhangs filled in with biotinylated nucleotides, and then free blunt ends were ligated. After ligation, crosslinks were reversed and the DNA purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was then sheared

to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The libraries were sequenced on an Illumina HiSeq X (rapid run mode).

Scaffolding the *Apalone* and *Staurotypus* assemblies with HiRise

The initial *de novo* assembly, shotgun reads, Chicago library reads, and Dovetail Hi-C library reads were used as input data for HiRise v.2016, a software pipeline designed specifically for using proximity ligation data to scaffold genome assemblies (Putnam et al. 2016). An iterative analysis was conducted. First, Shotgun and Chicago library sequences were aligned to the draft input assembly using a modified SNAP read mapper (http://snap.cs.berkeley.edu). The separations of Chicago read pairs mapped within draft scaffolds were analyzed by HiRise to produce a likelihood model for genomic distance between read pairs, and the model was used to identify and break putative misjoins, to score prospective joins, and make joins above a threshold. After aligning and scaffolding Chicago data, Dovetail Hi-C library sequences were aligned and scaffolded following the same method. After scaffolding, shotgun sequences were used to close gaps between contigs. Because the HiRise *Apalone* assembly thus obtained lacked the sex chromosome, a Z/W scaffold was identified by its homology to chicken GGA-15 (Kawagoshi et al. 2009; Badenhorst et al. 2013) from a publicly available fragmentary *Apalone* assembly (GCA_000385615.1) after scaffolding it using Ragtag2 v.2.1.0 followed by an Omni-C scaffolding as described by (Alonge et al. 2022). The quality of the resulting scaffolded- GCA_000385615.1 assembly was lower than the HiRise *de novo* genome assembly (Supplemental Table S4), such that only the Z scaffold was lifted and added to the *de novo* HiRise assembly for the comparative genomic analyses.

Curation of the Hi-C assembly

ASP and STR assembled genomes were manually curated using Hi-C and the Juicer/3D-DNA pipeline described in (Dudchenko et al. 2017) with default parameters. The curation was performed with Juicebox v.1.11.08 (Robinson et al. 2018). During this curation process some scaffolds were reoriented and superscaffolds were fragmented into smaller scaffolds. The criteria used to do the manual curation included: centromeric contacts position, contacts outside the diagonal, and HSBs analysis. To accelerate the process, the pre-curated assemblies were wrapped with the script wrap-fasta-sequence.awk from 3D-DNA utils package.

Supplemental Table S1: Read number and length from the sequencing of TruSeq, Chicago and Hi-C libraries.

		Staurotypus	*Apalone*
Illumina shotgun sequencing			
	Read length	**2x125 bp**	**2x400bp**
Library 1 (million reads)		524.8	439.4
Library 2 (million reads)		428.5	281
	Read length	**2x100 bp**	
Male Library (million reads)		320.1	
Female Library (million reads)		362.8	
Combined genome coverage (100-125 bp pairs)		72.1 ×	110.8 ×
Chicago library sequencing			
	Read length	**2x100 bp**	**2x150 bp**
Library 1 (million reads)		91	106
Library 2 (million reads)		83	130
Library 3 (million reads)		88	98
Library 4 (million reads)		90	
Combined genome coverage (1-100 kb pairs)		37.42 ×	30.53 ×
Hi-C library and sequencing			
	Read length	**2x150 bp**	**2x150 bp**
Library 1 (million reads)		231	136
Library 2 (million reads)		281	149
Library 3 (million reads)			132
Combined genome coverage (10-10,000 kb pairs)		4,051.13 ×	28,455.65 ×

Supplemental Table S2: Turtle genomes assembled in this study or compared to the new assemblies as described in the text, in alphabetical order.

Species	Common name	NCBI accession number	Source
Apalone spinifera	**Spiny softshell turtle**	**PRJNA837702**	**This study**
Caretta caretta	Loggerhead sea turtle	GCF_023653815.1	(Chang et al. 2023)
Carettochelys insculpta	Pig-nosed turtle	GCA_033958435.1	(Li et al. 2024)
Chelonia mydas	Green sea turtle	GCF_015237465.2	(Bentley et al. 2023)
Chrysemys picta	Painted turtle	GCF_000241765.5	(Lee et al. 2020)
Dermochelys coriacea	Leatherback sea turtle	GCF_009764565.3	(Bentley et al. 2023)
Gallus gallus	Chicken	GCA_016699485.1	(Warren et al. 2023)
Gopherus evgoodei	Goode's thornscrub tortoise	GCF_007399415.2	(Rhie et al. 2021)
Gopherus flavomarginatus	Bolson tortoise	GCF_025201925.1	(Rhie et al. 2021)
Malaclemys terrapin	Diamondback terrapin	GCF_027887155.1	(Rhie et al. 2021)
Mauremys mutica	Asian yellow pond turtle	GCF_020497125.1	(Liu et al. 2022)
Mauremys reevesii	Chinese pond turtle	GCF_016161935.1	(Liu et al. 2021a)
Pelochelys cantorii	Asian giant softshell turtle	GCA_032595735.1	(Liu et al. 2023)
Pelodiscus sinensis	Chinese softshell turtle	GCF_000230535.1	(Wang et al. 2013)
Rafetus swinhoei	Yangtze giant softshell turtle	GCA_019425775.1	(Ren et al. 2022)
Staurotypus triporcatus	**Mexican musk turtle**	**PRJNA1021228**	**This study**
Trachemys scripta elegans	Red-eared slider turtle	GCA_013100865.1	(Simison et al. 2020)

Supplemental Table S3: Chromosome homology between chicken (*Gallus gallus* - GGA) and turtles [*Apalone spinifera* (ASP), *Trachemys scripta* (TSC), and *Pelodiscus sinensis* (PSI)], based on genome sequencing, whole chromosome painting (WCP), or BAC in situ hybridization, from this study, O'Connor et al. 2018, Kasai et al. 2012, and Matsuda et al. 2005. Red font indicates few discrepancies likely due to differences between studies in the nomenclature of similar size chromosomes in turtles.

GGA (this study)	ASP (this study)	GGA (O'Connor et al. 2018)	Signal of GGA-WCP OR *GGA-BAC* hybridization in ASP (from O'Connor et al. 2018)	GGA WCP (Kasai et al. 2012)	TSC signal (Kasai et al. 2012)	GGA WCP (Matsuda et al. 2005)	PSI signal (Matsuda et al. 2005)
1	1	1	Weak	1	1	1	1
2	2	2	One chromosome	2	2	2	2
3	3	3	One chromosome	3	3	3	3
4	5 + 17	4	Two chromosomes	4	5 + 7	4	4
5	4	5	Weak	5	4q	5	5
6	8	6	One chromosome	6	7q		
7	7	7	One chromosome	7	11		
8	15b	8	One chromosome	8	8q		
9	12	9	One chromosome				
10	14	10	Large signal in 10, small				
11	15	11	One chromosome				
12	11	12	*Attached to 13*				
13	17q	13	*Attached to 12*				
14	16	14	*Clear on one similarly sized chromosome*				
15	32+ZW	15	*Clear on one similarly sized chromosome*				
16		16	*No result*				
17	25	17	*Clear on one similarly sized chromosome*				
18	9	18	*Clear on one similarly sized chromosome*				
19	27	19	*Clear on one similarly sized chromosome*				
20	10	20	*No result*				
21	23	21	*Clear on one similarly sized chromosome*				
22	31	22	*No result*				
23	26	23	*Clear on one similarly sized chromosome*				
24	9b	24	*Clear on one similarly sized chromosome*				
25	21	25	*Clear on one similarly sized chromosome*				
26	19	26	*Clear on one similarly sized chromosome*				
27	13	27	*Clear on one similarly sized chromosome*				
28	22	28	*Clear signal on one, similarly sized*				
34	1	29-38	Paint not successful in				
Z	6	Z	One autosome	Z	6	Z	6
W	6	W	No signal				

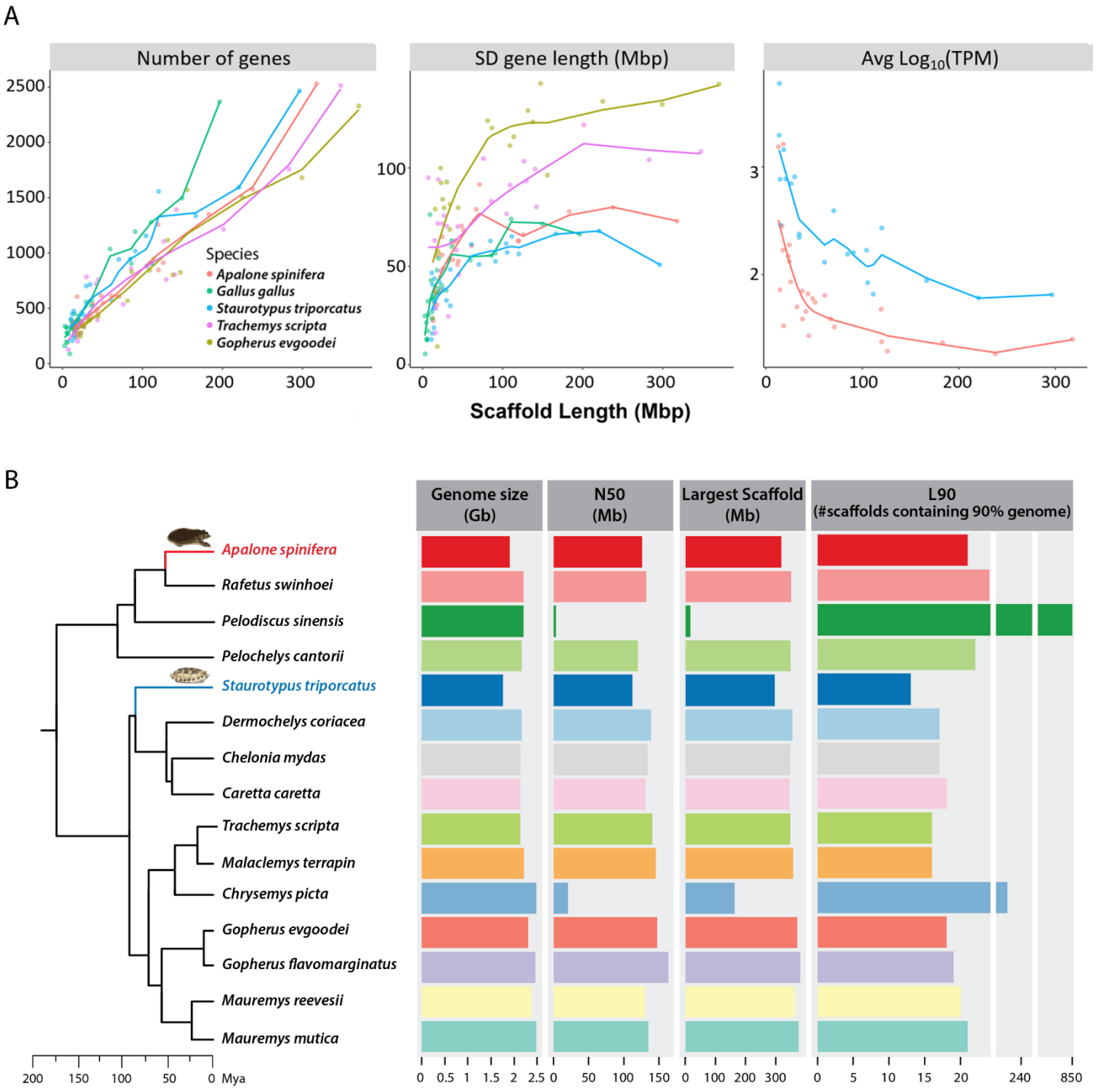
Supplemental Table S4: Quality comparison between our HiRise *Apalone spinifera* assembly and a publicly available fragmentary *A. spinifera* assembly (GCA_000385615.1) scaffolded here using Ragtag2 v.2.1.0 followed by an Omni-C scaffolding as described by (Alonge et al. 2022) as described in the text.

	***de novo Apalone spinifera* (Dovetail HiRise)**		***Apalone spinifera* (scaffolded GCA_000385615.1)**	
	Mbp	No. scaffolds	Mbp	No. scaffolds
Total	1904.79	6146	1946.49	132544
N50	120.19	5	75.58	7
N90	24.44	20	62.97	26
L50	5		7	
L90	20		26	
N_count	22.08		68.50	
Gaps	0.22		0.69	
Average	0.31		0.01	
Largest	317.84		240.20	

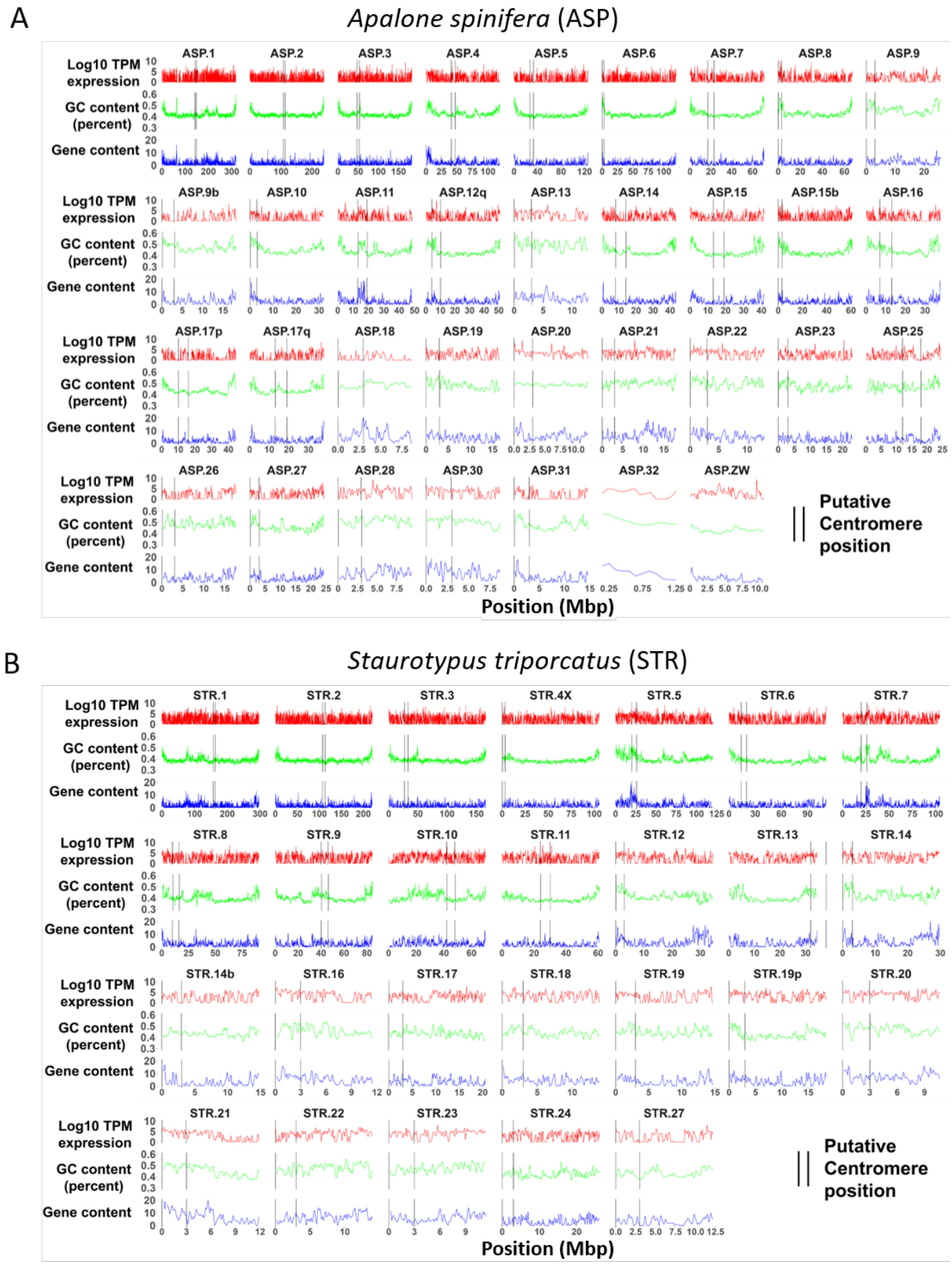
Supplemental Table S5: Quality metrics of Hi-C data per species.

Metric	Human	African Elephant	Tasmanian Devil	Platypus	Chicken	Emu	Softshell turtle	Musk turtle
Raw read pairs	200,000,000	422,511,000	415,946,000	200,000,000	176,543,289	211,025,822	118,849,394	281,312,029
Trimmed read pairs	188,521,812	401,429,000	343,209,000	181,816,000	116,957,000	202,004,662	96,762,998	235,889,173
Uniquely mapped read pairs	159,445,487	357,906,000	213,674,000	143,299,478	91,697,153	189,078,494	63,775,530	219,764,560
Self-circle (% relative uniquely mapped)	0.04	0.18	0.08	0.1	0.2	0.10	0.12	0.07
Dangling-end (% relative uniquely mapped)	1.31	0.13	0.06	21.49	20.2	18.98	8.86	31.3
Error (% relative uniquely mapped)	0.01	0.03	0.02	0.38	0.3	0.39	0.39	0.21
Extra dangling-end (% relative uniquely mapped)	4.27	0.79	0.88	20.61	21.73	41.60	31.58	37.48
Too short (% relative uniquely mapped)	5.82	4.52	3.35	10.9	4.65	12.03	18.93	14.57
Too large (% relative uniquely mapped)	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.00
Duplicated (% relative uniquely mapped)	1.17	8.98	7.92	6.2	10.62	16.13	9.26	15.96
Random breaks (% relative uniquely mapped)	0.02	0.23	0.1	3.09	1.55	7.23	6.34	0.61
Total valid read pairs	115,324,903	291,377,452	157,886,926	65,702,542	44,048,141	117,547,318	55,867,986	141,792,321
Average genome coverage	5.79	14.56	7.89	4.9	6.20	13.78	4.02	16.21
Total valid (% relative to Raw)	57.66	68.96	37.96	32.85	24.95	55.70	47.01	50.40
Total valid (% relative to Trimmed)	61.17	72.59	46.00	36.14	37.66	58.19	57.74	60.11
Total valid (% relative to Mapped uniquely)	72.33	81.41	73.89	45.85	48.04	62.17	87.60	64.52
SOURCE	Rao et al. (2014)	Álvarez-González et al. (2022)	Álvarez-González et al. (2022)	Zhou et al. (2021)	Fishman et al. (2019)	Liu et al. (2021)	This paper	This paper

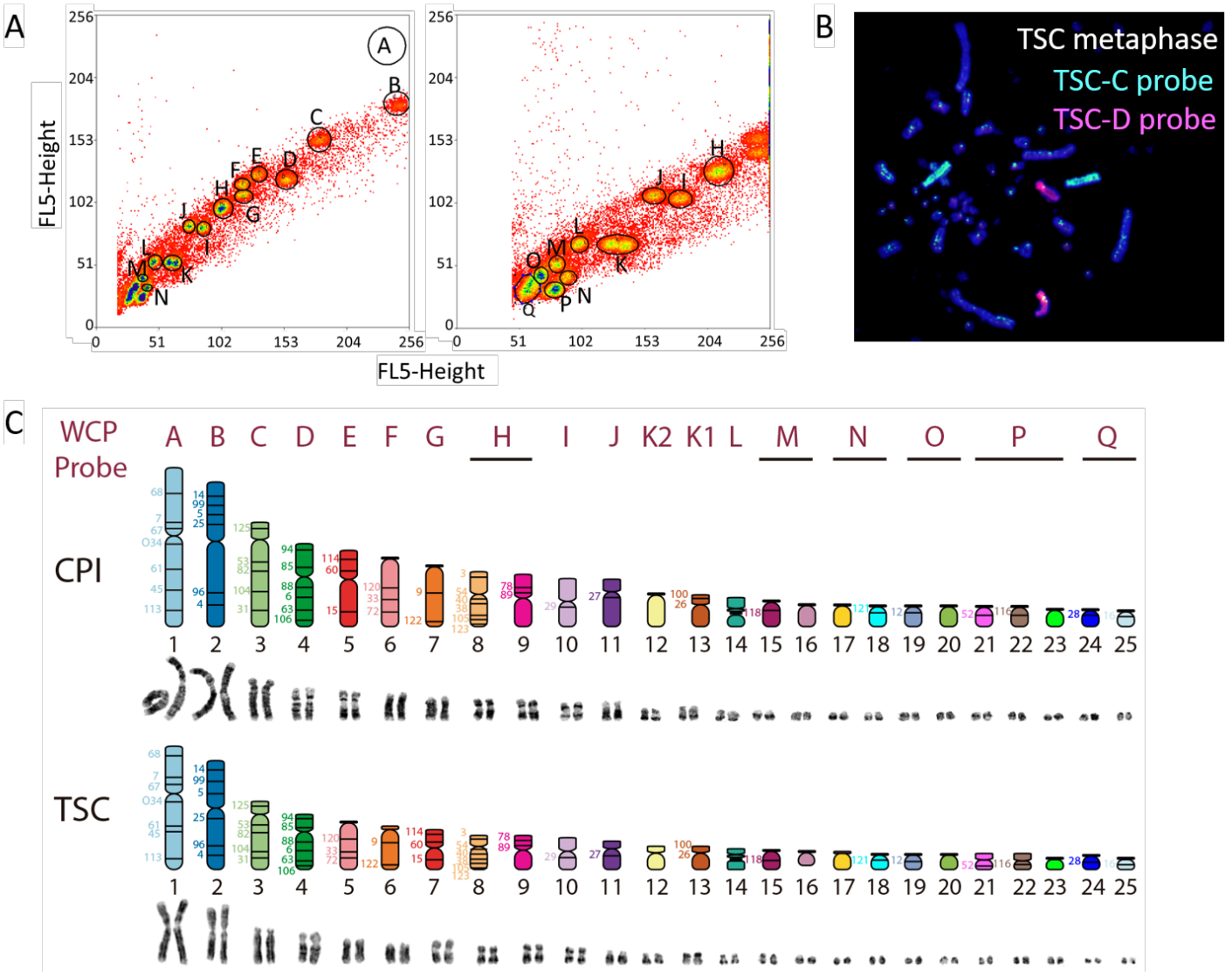
Supplemental Figure S1: (A) Total number of genes and average gene length by chromosome length in three turtles and chicken: *Apalone spinifera* (this study), *Staurotypus triporcatus* (this study), *Trachemys scripta elegans* (Simison et al. 2020), and *Gallus gallus* (Warren et al. 2023). **(B)** Comparative quality data for *Apalone spinifera* (this study), *Staurotypus triporcatus* (this study), and 13 other published genome assemblies cited in the text. Tree based on (Thomson et al. 2021).



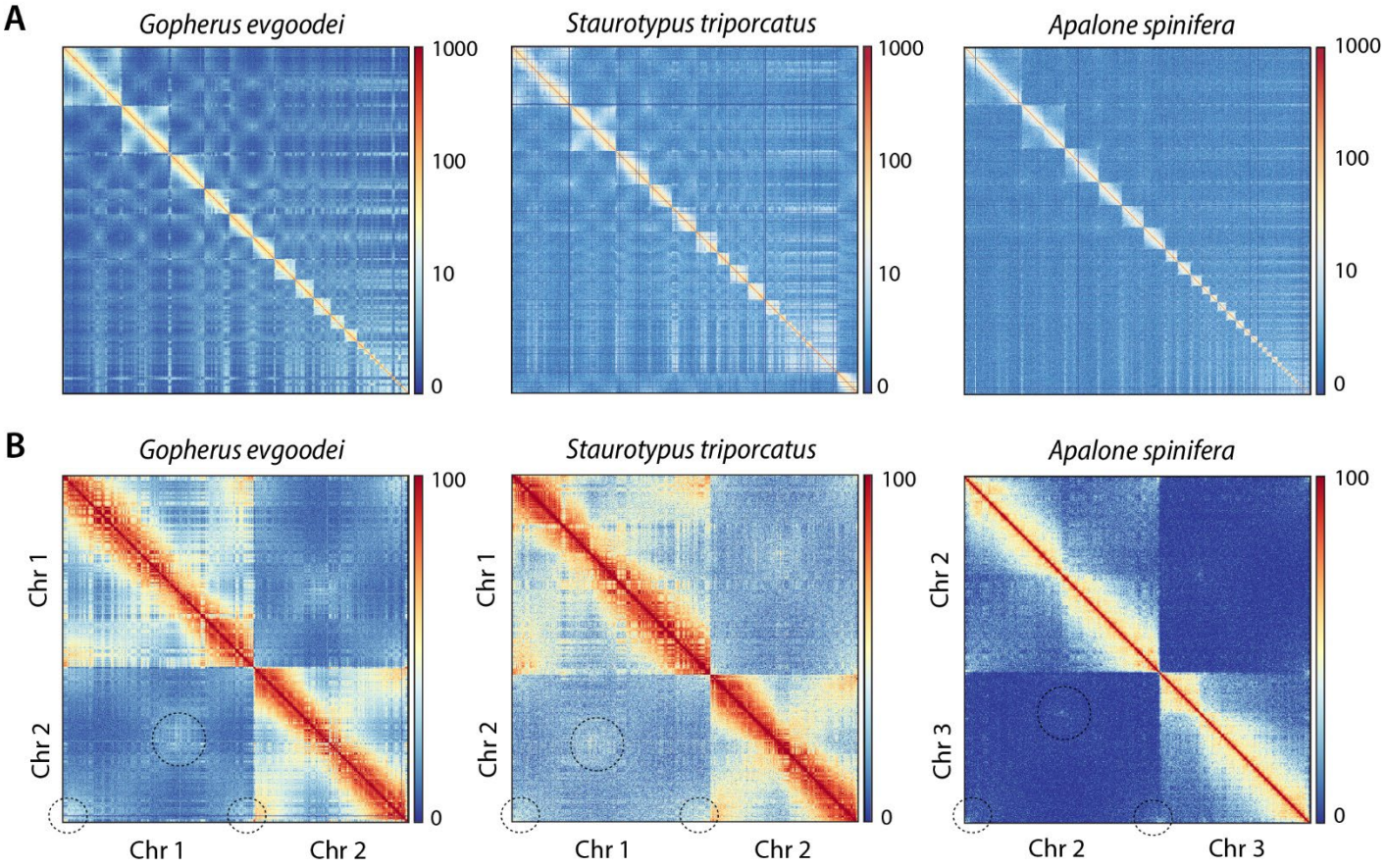
Supplemental Figure S2: Chromosome-wise genome statistics. Chromosome-wise gene content, GC content, and gene expression (Log_{10} normalized TPM) for (A) *Apalone spinifera* (ASP) and (B) *Staurotypus triporcatus* (STR).



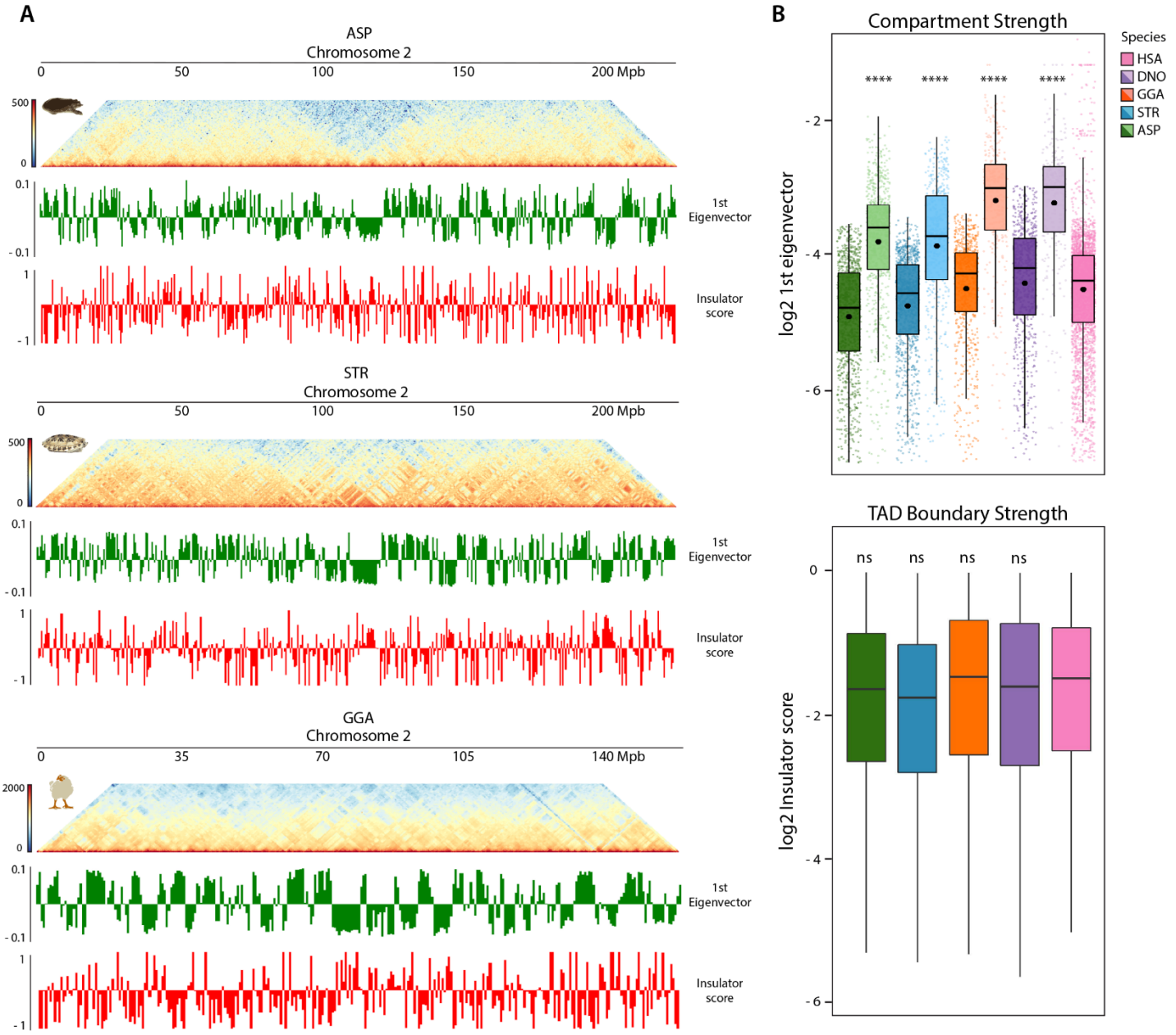
Supplemental Figure S3: (A) Flow sorting of *Trachemys* chromosomes via flow cytometry generated 18 probes, named A-Q. **(B)** Example of whole chromosome painting onto *Trachemys picta* metaphase chromosomes using the probes obtained by flow sorting, which permits identifying cytogenetically each flow sorted chromosome or group of chromosomes. **(C)** *Trachemys* and *Chrysemys* karyotypes (Montiel et al. 2016) and ideograms of chromosomes assigned by the combination of whole chromosome painting (WCP) data and BAC-FISH data from this study (using BAC clones from a *Chrysemys* BAC library) and from the literature, [e.g. (Badenhorst et al. 2015; Lee et al. 2020)], as described in the main text.



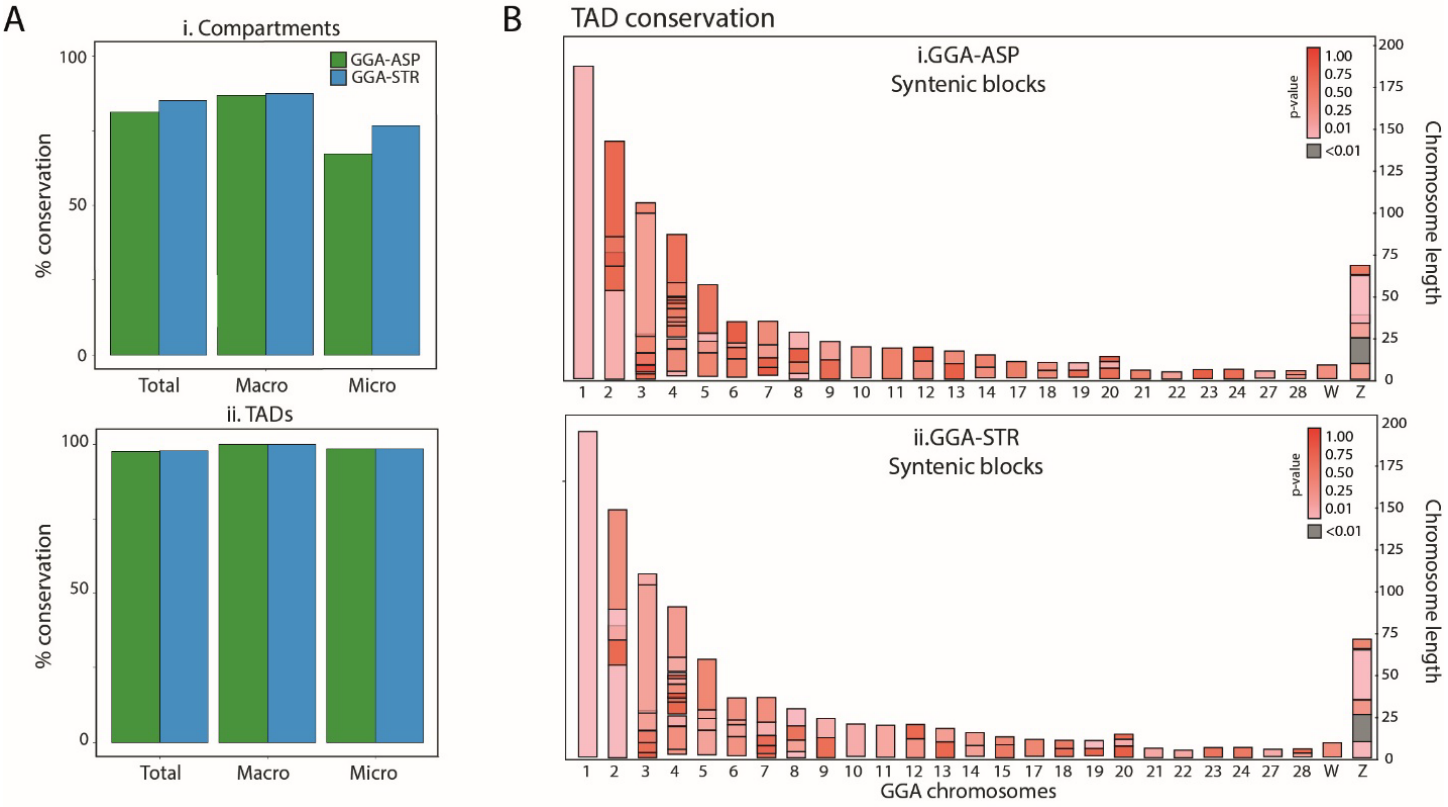
Supplemental Figure S4: (A) Whole-genome Hi-C contact maps for Sinaloan Thornscrub Tortoise [*Gopherus evgoodei,*(Rhie et al. 2021)], Mexican Giant Musk Turtle (*Staurotypus triporcatus*, this study) and Spiny Softshell Turtle (*Apalone spinifera*, this study). **(B)** Chromosome-specific Hi-C contact maps representing a pair of macro chromosomes for the same three species. Both telomeric and centromeric interactions are highlighted by circles.



Supplemental Figure S5: Genome compartmentalization in turtles. **(A)** Chromosome 2 region-specific 500 kbp heatmaps, first eigenvector (green), insulator score (red) for Spiny Softshell Turtle [*Apalone spinifera* (ASP), this tudy], Mexican Giant Musk Turtle [*Staurotypus triporcatus* (STR), this study], and chicken [*Gallus gallus* (GGA), (Fishman et al. 2019)]. Positive and negative eigenvalues denote A and B compartments, respectively. Absolute eigenvalues reflect compartment definition (compartment strength) whereas insulation scores reflect how well-defined TADs are (points of no interaction would have a score ≈0). **(B)** Boxplots depicting $\log_2$-transformed first eigenvector values (i.e. compartment strength) and insulator score values (TAD boundary strength) for human [*Homo sapiens* (HSA), (Rao et al. 2014)], Emu [*Dromaius novaehollandiae* (DNO), (Liu et al. 2021b)], chicken [*Gallus gallus* (GGA), (Fishman et al. 2019)], Spiny Softshell Turtle [*Apalone spinifera* (ASP), this study], and Mexican Giant Musk Turtle [*Staurotypus triporcatus* (STR), this study]. Darker and lighter color tones in the top B panel denote values for macro- and micro-chromosomes, respectively. Asterisks represent statistically significant different interactions between species (two-tailed *t*-test, ***p < 0.001). ns: non-significant.



Supplemental Figure S6: Conservation of chromatin structure in sauropsids. (A) Percentage of compartments (upper panel) and TADs (lower panel) conserved between chicken and *Apalone* and between chicken (green) and *Staurotypus* turtles (blue), genome-wide (total) and for macro and micro-chromosomes separately. *Gallus gallus* (GGA), *Apalone spinifera* (ASP), and *Staurotypus triporcatus* (STR). **(B)** Chicken chromosome ideograms color-coded accordingly to the conservation of TADs of syntenic blocks between *Gallus* and *Apalone* (upper panel) and between *Gallus* and *Staurotypus* (lower panel), expressed as p-value scores (statistically significant p-values are denoted in gray).



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