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Physical Mapping and Refinement of the Painted Turtle Genome (*Chrysemys picta*) Inform Amniote Genome Evolution and Challenge Turtle-Bird Chromosomal Conservation

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Abstract

Comparative genomics continues illuminating amniote genome evolution, but for many lineages our understanding remains incomplete. Here, we refine the assembly (CPI 3.0.3 NCBI AHGY00000000.2) and develop a cytogenetic map of the painted turtle (*Chrysemys picta*—CPI) genome, the first in turtles and in vertebrates with temperature-dependent sex determination. A comparison of turtle genomes with those of chicken, selected nonavian reptiles, and human revealed shared and novel genomic features, such as numerous chromosomal rearrangements. The largest conserved syntenic blocks between birds and turtles exist in four macrochromosomes, whereas rearrangements were evident in these and other chromosomes, disproving that turtles and birds retain fully conserved macrochromosomes for greater than 300 Myr. C-banding revealed large heterochromatic blocks in the centromeric region of only few chromosomes. The nucleolar-organizing region (NOR) mapped to a single CPI microchromosome, whereas in some turtles and lizards the NOR maps to nonhomologous sex-chromosomes, thus revealing independent translocations of the NOR in various reptilian lineages. There was no evidence for recent chromosomal fusions as interstitial telomeric-DNA was absent. Some repeat elements (CR1-like, Gypsy) were enriched in the centromeres of five chromosomes, whereas others were widespread in the CPI genome. Bacterial artificial chromosome (BAC) clones were hybridized to 18 of the 25 CPI chromosomes and anchored to a G-banded ideogram. Several CPI sex-determining genes mapped to five chromosomes, and homology was detected between yet other CPI autosomes and the globally nonhomologous sex chromosomes of chicken, other turtles, and squamates, underscoring the independent evolution of vertebrate sex-determining mechanisms.

Key words: physical molecular cytogenetic BAC clone mapping, chromosomal rearrangements, genome and chromosome evolution, translocations and inversions, nonmodel vertebrates, turtles, chicken, human, temperature-dependent and genotypic sex determination.

Introduction

Novel and recalcitrant questions in biology are elucidated at an increasing pace thanks to the development of new genomic resources in nonmodel organisms (Janes et al. 2008), such as the recent release of several chelonian (turtle) genomes: The western painted turtle *Chrysemys picta* (CPI) (Shaffer et al. 2013), the Chinese softshell turtle *Pelodiscus sinensis*, and the sea turtle *Chelonia mydas* (Wang et al. 2013). These turtle genomes complement recent sequencing efforts on other major reptilian groups including lizards (Alfoldi et al. 2011), crocodilians (St John et al. 2012; Green et al. 2014), and snakes (Castoe et al. 2013; Vonk et al. 2013). Phylogenetic analyses enabled by these chelonian genomes strongly support the hypothesis that turtles are sister to

Archosaurs (birds and crocodilians) (Chiari et al. 2012; Crawford et al. 2012; Deakin et al. 2013; Shaffer et al. 2013), a result of paramount importance as the accurate placement of turtles in the tree of life is essential for the reconstruction of the evolutionary history of vertebrate traits and genomes.

A major aspect of genome organization influencing genome function and evolution is its compartmentalization into chromosomes, because changes in the synteny of genes and gene blocks alter their regulatory environment (Ahituv et al. 2005), affecting transcription (De et al. 2009) and adaptation (Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008; Loxdale 2010). Genome organization varies among taxa and coevolves with other traits: An example is the coevolution of chromosome number and sex determination in turtles (Valenzuela and Adams 2011), or the location and types of repeat elements and evolutionary breakpoints of chromosomes prone to rearrangements (Flint et al. 1994; Azzalin et al. 2001; Ruiz-Herrera et al. 2005). Additionally, karyological evolution is linked to lineage diversification in a variety of organisms, including reptiles (Olmo et al. 2002; Ayala and Coluzzi 2005; Olmo 2005; Hoffmann and Rieseberg 2008). Thus, evolutionary and functional genomics benefit not only from sequence data but also from cytogenetic information that places DNA sequences in their physical and phylogenetic context to enable evolutionary inferences across species.

In particular, comparative cytogenetic and sequence analyses have illuminated many aspects of vertebrate genome evolution (Deakin and Ezaz 2014) although much remains to be learned. For instance, the sequencing and physical mapping of the chicken genome revealed the homology between bird and human chromosomes (Nanda et al. 2000; Schmid et al. 2000), and the high conservation of the avian genome previously attributed to the scarcity of repeat elements (Backstrom et al. 2008) was later confirmed by additional genome analyses (Dalloul et al. 2010; but see Griffin et al. 2007). Sequencing of outgroup genomes is also important for phylogenomics. For example, the opossum and platypus genomes revealed shared and unique genomic components in monotremes, birds, and therian mammals (Mikkelsen et al. 2007; Warren et al. 2008), whereas genome evolution in teleosts and gnathostomes is anchored by the coelacanth and lamprey genomes (Kasahara et al. 2007; Amemiya et al. 2013; Smith et al. 2013). Comparative approaches have also permitted the reconstruction of ancestral karyotypes in lineages such as primates, marsupials, amniotes, tetrapods, and vertebrates (De Leo et al. 1999; Richard et al. 2003; Kemkemer et al. 2006, 2009; Kohn et al. 2006; Nakatani et al. 2007; Stanyon et al. 2008; Uno et al. 2012; Deakin et al. 2013; Romanov et al. 2014), among others. Although sequence comparisons between the recently sequenced turtle genomes and those of other vertebrates revealed a less prominent GC-rich isochore structure in turtles than in mammals and birds (Shaffer et al. 2013), we know less about the chromosomal rearrangements that have accrued during chelonian evolution.

Turtles are a reptile group reported to have highly conserved karyotypes when compared with lizards and snakes in terms of the number, morphology, and G-banding pattern of their chromosomes (Bickham 1981; Olmo 2008). Within turtles, this conservation is greater in the suborder Criptodira—to which all newly sequenced turtles belong—relative to the suborder Pleurodira. Previous studies have consistently identified highly conserved homology between some turtle chromosomes and those of other vertebrates, most notably between the six largest turtle and chicken chromosomes (Matsuda et al. 2005), including CHICKEN-Z and *P. sinensis* turtle chromosome 6 (PELODISCUS-6) (Matsuda et al. 2005; Kawai et al. 2007); PELODISCUS-Z/W and CHICKEN-15 (Kawagoshi et al. 2009), and PELODISCUS-6 and *Elaphe quadrivirgata* snake chromosome 2 (ELAPHE-2) (Matsuda et al. 2005). Turtles resemble birds and lizards in the presence of microchromosomes, some of which may also represent ancient syntenies conserved since the rise of vertebrates 400 Ma (Burt 2002), but which are notably absent in mammals and crocodilians. Thus, more extensive analyses encompassing a larger portion of the turtle karyotypes are still needed to gain a comprehensive understanding of genome evolution in turtles and vertebrates.

Here, we present an improved genome assembly and the first physical BAC mapping of the painted turtle (CPI) genome, the first of any vertebrate with temperature-dependent sex determination (TSD), and a comparison with other vertebrates where information is available (mainly chicken and human). Importantly, we found evidence dispelling the full conservation of several purported syntenies while supporting the conservation of some significant vertebrate gene blocks and the occurrence of numerous chromosomal rearrangements over more than 300 Myr of vertebrate evolution. Specifically, we complement previous basic cytogenetic data for CPI (Killebrew 1977; De Smet 1978) with detailed G- and C-banded karyotypic and molecular cytogenetic information otherwise lacking for this emerging model species (Valenzuela 2009), including 1) the distribution of repeat elements, 18S rDNA and telomeres; 2) the first banded ideogram for this species (diagrammatic representation of the haploid chromosome set); and 3) the mapping of 61 sequenced BACs (some containing genes involved in sexual development). Additionally, using a new set of bioinformatic algorithms we obtained an improved genome assembly with fewer and larger scaffolds (see Supplementary Information) than the original released CPI genome (Shaffer et al. 2013). Our molecular cytogenetic data permit the refinement of the painted turtle genome assembly by anchoring scaffolds to chromosomes

and therefore provide the most detailed picture yet of the structure of turtle chromosomes.

Materials and Methods

Cell Culture, Chromosome Preparation, and Chromosome Banding

Primary fibroblast cell cultures for cytogenetic analyses were established using limb tissue from one male and one female CPI 6-month-old hatchling as well as the adult female whose genome was sequenced and reported in Shaffer et al. (2013). The sex of all individuals was assessed by gonadal inspection. Briefly, fibroblast cell cultures were established from collagenase (Sigma) digests and cultured using a medium which was composed of 50% RPMI 1640 and 50% Leibowitz media supplemented with 15% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic-antimycotic solution (Sigma). Cultures were incubated at 30 °C with no CO_2 supplementation (Badenhorst et al. 2013). Four hours prior to harvesting 10 μg/ml colcemid (Roche) was added to the cultures. Metaphase chromosomes were harvested after colcemid arrest (KaryoMAX; Invitrogen), hypotonic exposure, and fixed in 3:1 methanol:acetic acid following standard procedures (Ezaz et al. 2006; Martinez et al. 2008; Badenhorst et al. 2013). G- and C-banding followed conventional protocols (Seabright 1971; Sumner 1972). The distribution of NORs (the genomic region containing the genes for the 18S, 5.8S, and 28S ribosomal subunits) was investigated by silver staining (Ag-NOR) (Goodpasture and Bloom 1975), and by fluorescent in-situ hybrydization (FISH) using a turtle-specific 18S DNA fragment labeled by nick-translation and coprecipitated with salmon sperm DNA (Badenhorst et al. 2013). A telomeric probe containing the repeat motif (TTAGGG)n was generated and labeled by polymerase chain reaction, starting with $(TTAGGG)_4$ and $(CCCTAA)_4$ primers in the absence of template DNA (Ijdo et al. 1991).

BAC Clone Sequencing and Hybridization

Sets of randomly chosen clones from a CPI BAC library (library VMRC CHY3 produced by the Joint Genome Institute) were sequenced (through Illumina) as part of the turtle genome sequencing project (Shaffer et al. 2013), and others (12 BACs) were screened for the putative presence of genes in the turtle and vertebrate sex determination network (Valenzuela 2010; Valenzuela et al. 2013) and sequenced independently in full (454 platform) or in part (Sanger sequencing) to confirm the presence of genes of interest. These sequenced BACs (81 in total) were used to link cytogenetic and DNA sequence data, to refine the genome assembly, and to establish the syntenic relationships (relative genome position) of functional genes. BAC DNA (~1 μg) was extracted and labeled by standard nick-translation (Abbott Molecular) using either biotin or digoxingenin dUTPs (Roche) and coprecipitated with human cot-1 DNA and turtle cot-1 DNA. FISH was carried out using BAC, telomere, and 18S probes, by dehydrating the slides through an ethanol series followed by denaturing the chromosome preparations together with the probe-mix on a hot plate at 65 °C for 2 min, and hybridization took place overnight (two nights for 18S rDNA and telomere probes) in a humid chamber at 37 °C. Posthybridization washes were comprised of a first wash in 0.4 × Saline-Sodium Citrate (SSC)/0.3% Tween 20 for 2 min at 60°C, followed by a second wash in 2 × SSC/0.1% Tween 20 for 1 min at room temperature. Fluorochrome detection was performed with 4XT/relevant antibody in a 200-μl final volume at 37°C for 45 min. Slides were subsequently washed thrice in 4XT at 37 °C, counterstained with DAPI (6 μl DAPI 2 mg/ml in 50 ml 2 × SSC), and mounted using an antifade solution (Vectashield). Signals were assigned to specific chromosomes according to their morphology, size, and DAPI-banding. FISH was repeated after G-banding to improve anchoring of BAC sequences to chromosomal regions, and two to four BAC FISH was used to determine relative position within chromosomes. Images were taken with a Photometrics CoolSnap ES2 Digital Monochrome camera attached to an Olympus BX41 fluorescent microscope, and analyzed using CytoVision cytogenetic analysis system (Applied Imaging/Genetix).

BAC Bioinformatics for Cytogenetic Analysis

All 61 sequenced BACs were mapped to CPI's reference genome assembly 3.0.3 using Geneious v.6.1.6 (Kearse et al. 2012), as follows. First, Megablast was used to search for the complete BAC sequence within the annotated CPI genome. The annotations in the CPI genome were used to identify putative protein-coding genes that co-occur on the same genomic scaffold as the mapped BAC (61 in total). Genomic scaffolding was improved in this study (assembly 3.0.3) with respect to the original genome assembly 3.0.1 (Shaffer et al. 2013) (see Supplementary information). The genomic location of those genes (1,425 genes in total) in the human and chicken genomes was determined from a direct search in the NCBI (National Center for Biotechnology Information) Database (Assemblies: homSapGRCh38, galGal4), and information in other vertebrates was obtained from the published literature.

Results and Discussion

Cytogenetic Data and Ideogram

A combination of classic and molecular cytogenetics allowed a deeper characterization of the CPI karyotype than was previously reported for this species (Killebrew 1977; De Smet 1978), and included G- and C-banding, Ag-NOR (nucleolar-organizing region), 18S rRNA, and telomere DNA mapping (figs. 1 and 2). At least ten cells per individual were analyzed

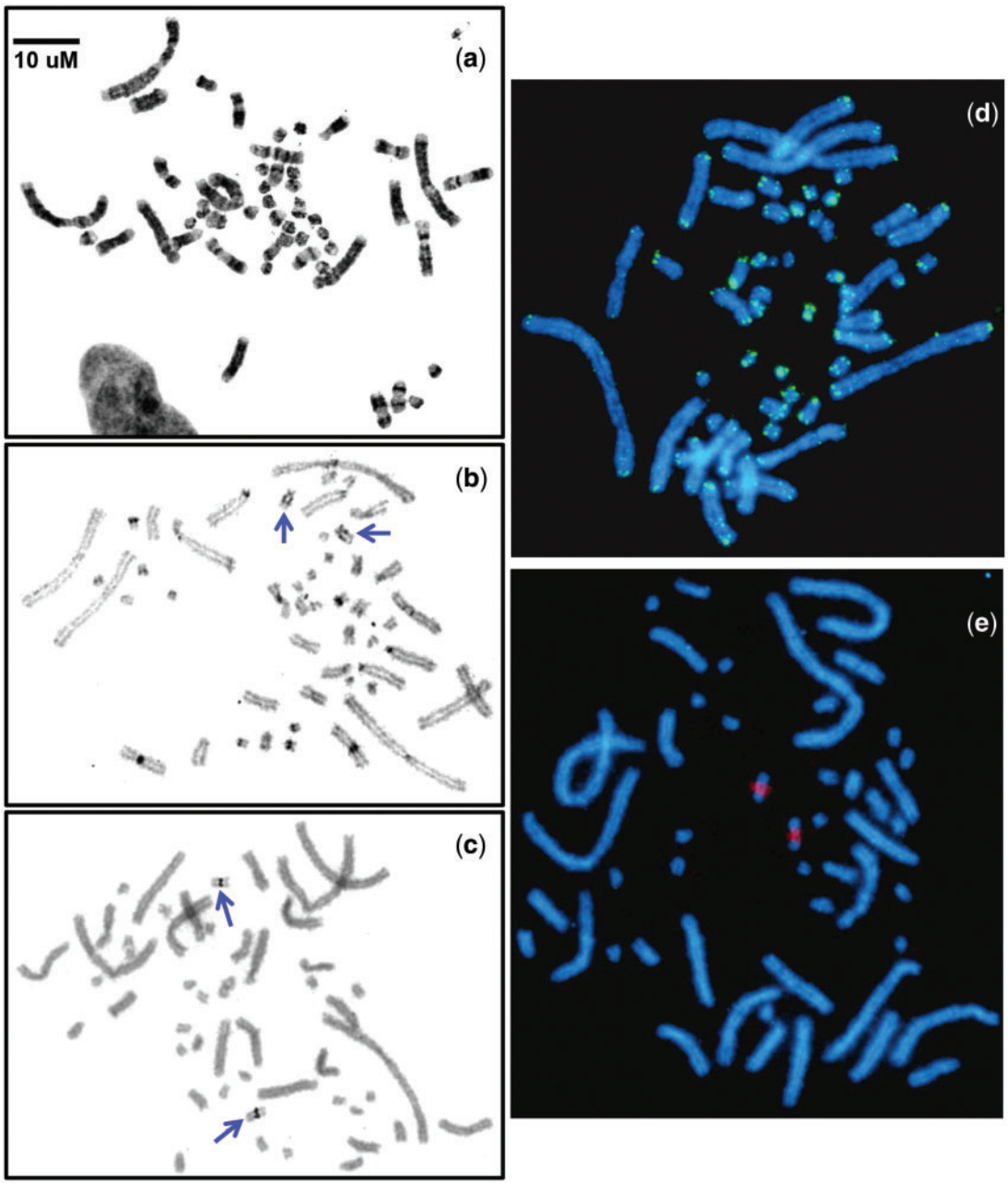

Fig. 1.— G-banded (*a*), C-banded (*b*), and Ag-NOR stained (*c*) metaphase chromosomes of CPI, and the distribution of telomeric DNA (*d*) and 18S rDNA repeats (*e*) on CPI metaphase spreads. Arrows indicate C-positive interstitial bands (*b*) and NOR localization (*c*).

for all cytogenetic procedures. The G-banded ideogram of the haploid genome of CPI presented here depicts 189 defined G-bands (dark AT-rich heterochromatin and light CG-rich euchromatic bands; fig. 2). The smaller microchromosomes of CPI were nearly indistinguishable from each other by shape and were ordered by approximate size and G-banding pattern where possible. Ours is the first banded ideogram developed for any turtle and for any vertebrate with TSD and lacking sex chromosomes (Valenzuela et al. 2014).

All three individuals examined had $2N = 50$ chromosomes with 26 macrochromosomes and 24 microchromosomes (4 metacentric, 18 submetacentric, 28 acrocentric), a result that differs slightly from the 24 macrochromosomes and 20 metacentric/submetacentric chromosomes described by De Smet (1978), but in agreement with Killebrew (1977). The subtle difference from the report by De Smet (1978) could be due to individual or population differences, but is more likely due to the difficulty of unambiguously identifying the morphology of

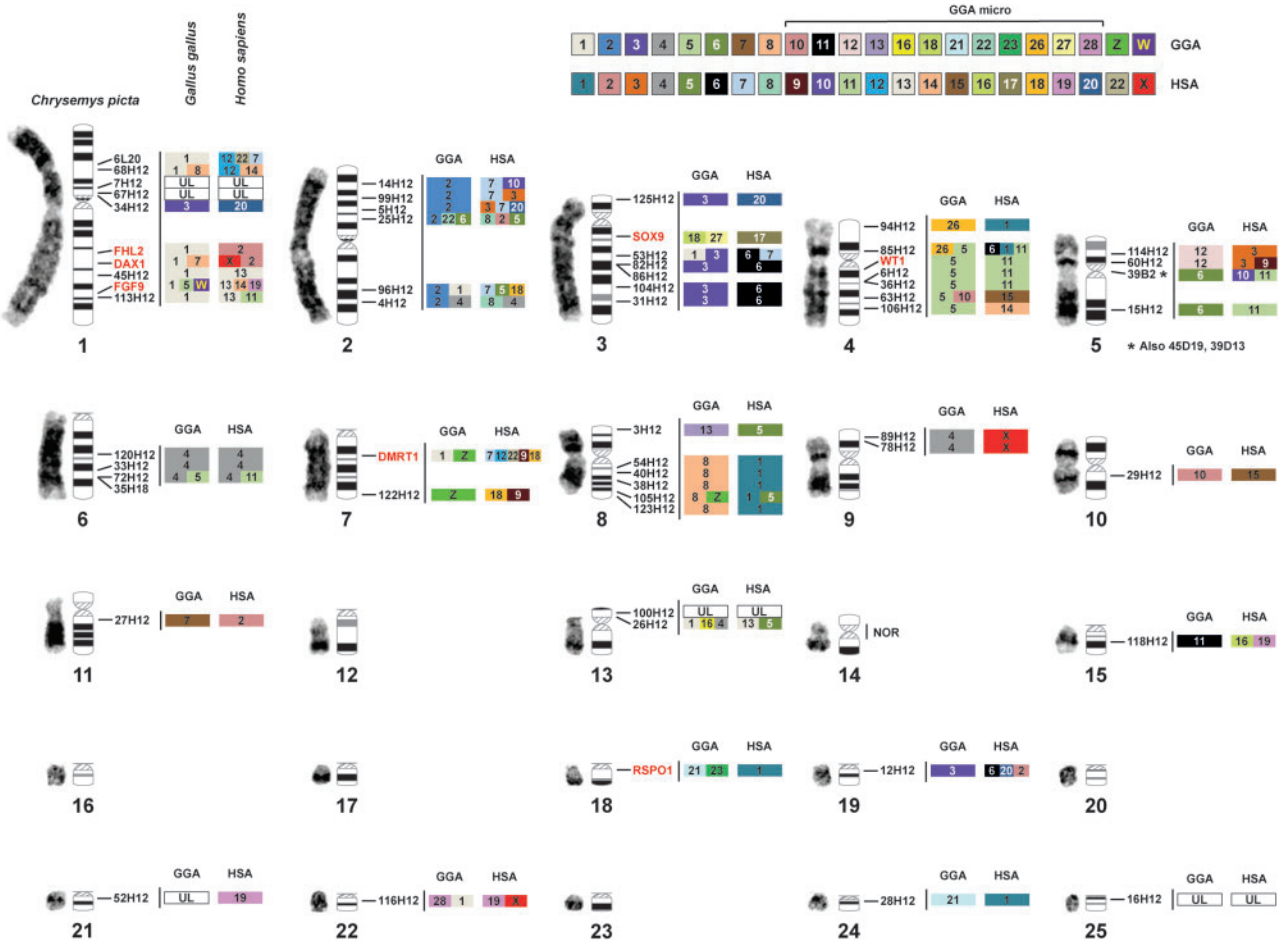

Fig. 2.— CPI GTG-banded karyotype and ideogram showing the physical location of FISH-anchored BAC clones, with sex-linked BACs in red, along with the homology of gene blocks to chicken (GGA) and human (HSA) chromosomes, depicted as colored blocks, and with the NOR location on CPI 14. "UL" indicates uncharacterized loci, that is, CPI BACs that did not map to annotated scaffolds and thus homology to GGA and HSA was precluded. Note that BACs 45D19, 35H18, 39D13 colocate with 39B2 in CPI-5, and BAC 6L20 shows some hybridization to this region as well, likely due to the presence of shared repeat sequences (see supplementary table S2, Supplementary Material online).

turtle microchromosomes, because they may appear telocentric, making the position of the centromere difficult to discern (De Smet 1978).

The C-positive material representing constitutive heterochromatin is largely restricted to the centromeric regions with clearly observable blocks only visible in some chromosome pairs. Variation in the abundance of heterochromatin in the centromeric regions among chromosomes is observed by C-banding in a variety of taxa including humans, and reflects differences in the types and number of repeat elements and satellite DNA (Sumner 2003). There is, however, interstitial heterochromatin visible in chromosome pair 14 (fig. 1*b*) that corresponds to the NOR (containing genes encoding the three major ribosomal RNA subunits—18S, 5.8S, and 28S [Shaw and McKeown 2011]). Indeed, a single interstitial NOR was detected on chromosome pair 14 by both silver staining, which detects active NORs, and 18S rDNA FISH, which detects active and inactive NORs (fig. 1*c* and *e*), corresponding to this C-positive band. These results agree with a previous report using silver staining only (Bickham and Rogers 1985). Interstitial NORs are not ubiquitous in turtles; only 12 of 28 investigated turtles display interstitial NOR sites, but they seem widespread in the subfamily Emydinae, to which CPI belongs (Bickham and Rogers 1985). C-positive blocks are also found in reptilian sex chromosomes (Kawai et al. 2007; Singh 2011; Badenhorst et al. 2013; Matsubara et al. 2014; Rojo et al. 2014) and may colocalize with the NOR in the W chromosome of some turtles (Kawai et al. 2007; Badenhorst et al. 2013) and lizards (Matsubara et al. 2014; Rojo et al. 2014). However, our BAC mapping data rule out the hypothesis that CPI-14 is homologous to any of these reptilian sex chromosomes, and instead suggest that the NOR has undergone translocations to multiple chromosomal locations independently in various reptilian lineages.

Telomeric repeats localized exclusively to the end of the chromosome arms (fig. 1*d*), as expected given that telomeres

play a key role in maintaining chromosome stability [(Bolzán and Bianchi 2006)]. Interstitial telomeres are indicative of past chromosomal rearrangements and evolutionary unstable genome regions (Ruiz-Herrera et al. 2008). The absence of interstitial telomeric sequences in CPI corresponds with previous reports in other turtles which also lack them (e.g., *Trachemys dorbigni* and *Chelonoidis donosobarrosi* [Martinez et al. 2009]), as well as tuatara (O'Meally et al. 2009). However, these data contrast with lizards where they are present, for example, in microchromosomes in *Pogona vitticeps* (Young et al 2013), macrochromosomes in *Iberolacerta monticola* (Rojo et al. 2014) or both in *Leiolepis* lizards (Srikulnath et al. 2011). However, additional data on the chromosomal location of telomeric sequences in a larger subset of turtle taxa are needed to test whether such contrasting patterns among these major reptilian lineages are generalizable.

BAC-Mapping and Bioinformatic Analysis

A total of 61 of the 81 fully sequenced BAC clones were successfully assigned to a unique location in the CPI ideogram (figs. 2 and 3). BAC clones were assigned to 12 of the 13 macrochromosomes and 7 of the 12 microchromosomes. Multicolor FISH was used to anchor BACs to microchromosomes and to macrochromosomes of similar size and shape. Thus, 19 of the 25 chromosome pairs have BAC markers assigned to them, and chromosome pair 14 is distinguishable by its C-positive block and by the localization of the NOR detectable by silver staining and/or 18S-FISH (fig. 1). This cytogenetic BAC mapping information was combined with bioinformatics analyses to refine the painted turtle genome assembly (see supplementary information, Supplementary Material online) and resulted in improved ultrascaffolds and chromosomal information. For instance, chromosomal AGPs were created and centromeres were positioned using the BAC maps, which localized 461 Mb of genomic DNA to 18 chromosomes. AGPs are "A Golden Path" description files of the components of each chromosome. This is the first chromosomal AGP produced for a turtle and the second for nonavian reptiles (Alfoldi et al. 2011). The improved genome sequence of CPI 3.0.3 was deposited in the DDBJ/EMBL/GenBank database (accession number AHGY00000000.2). There were a few mapped BACs containing DNA sequences with no annotation and are referred to as "uncharacterized loci" in the figures and tables.

An additional subset of 18 BAC clones produced too much background during FISH to permit accurate localization (fig. 3). Interestingly, these problematic BACs contained a large proportion of various repeat elements indicating that repeats are abundant and widespread throughout the turtle genome (supplementary table S2, Supplementary Material online; fig. 3*d*), consistent with the sequenced genome analysis (Shaffer et al. 2013). In contrast, the hybridization signal from four BACs enriched for CR1-like and Gypsy repeat sequences exhibited a clustered pattern in the centromeric region of five chromosome pairs, including a macrochromosome (CPI-5) and four microchromosomes (fig. 3*c*). These repeat elements are also shared by four additional BACs that map uniquely to the same region in CPI-5. These results indicate that unlike other simpler repeat types, these transposable elements are not randomly distributed in the CPI genome but instead predominate on five chromosome pairs. This is the first indication that turtle centromeric and pericentromeric regions are not uniform in their composition, similar to what is observed in chicken (Shang et al. 2010), humans, and other metazoans (Maddox et al. 2012; Fukagawa and Earnshaw 2014). These results are also important because these repeats may affect the evolution and regulation of these genomic regions disproportionately (Kudla et al. 2006), and transposable elements have played a significant role in the evolution of other vertebrate genomes, such as in mammals (Mikkelsen et al. 2007).

Bioinformatic analyses of the BAC sequences and the CPI genome scaffolds to which they map permitted the first assessment of homology between the painted turtle chromosomes and those of chicken (*Gallus gallus* [CHICKEN]) and human (*Homo sapiens* [HUMAN]), as well as a few other reptiles where partial genome or cytogenetic information was available (fig. 4, table 1), specifically the turtle *P. sinensis* (PELODISCUS), the snake *E. quadrivirgata* (ELAPHE), and several lizards (*Varanus salvator* [VARANUS], *Leiolepsis reevesii*, (LEIOLEPSIS) *Po. vitticeps* [POGONA]) (Matsuda et al. 2005; Matsubara et al. 2006; Srikulnath et al. 2013; Young et al. 2013). In general, our data challenge the previously reported conservation of macrochromosomes between birds and turtles (Matsuda et al. 2005; Kasai et al. 2012). Specifically, by using a much larger gene data set from our BAC clones and the scaffolds to which they map in the CPI genome (1,425 genes) we identified numerous putative chromosomal rearrangements that passed undetected when using fewer markers in other turtles (e.g., 57 genes in Matsuda et al. [2005]). Additionally, our data set allowed inferences of homologies for over 70% of the turtle chromosome pairs (18 of 25) for the first time, including intermediate sized and microchromosomes. Specifically, we found that CPI and chicken macrochromosomes 1, 2, and 3 represent the highest conserved synteny. However, this is not a fully conserved synteny because they contain regions orthologous to at least four and six chicken chromosomes; this number is a conservative estimate as our BAC coverage is not complete on the CPI genome. Furthermore, CPI-4 contains larger gene block regions that are orthologous to at least five chicken chromosomes compared with the smaller blocks that interrupt the synteny of CPI-1, CPI-2 and CPI-3 and CHICKEN-1, CHICKEN-2 and CHICKEN-3, respectively. The gene blocks identified in CPI-1 to CPI-4 are orthologous to numerous human chromosomes including HUMAN-X (figs. 2 and 4,

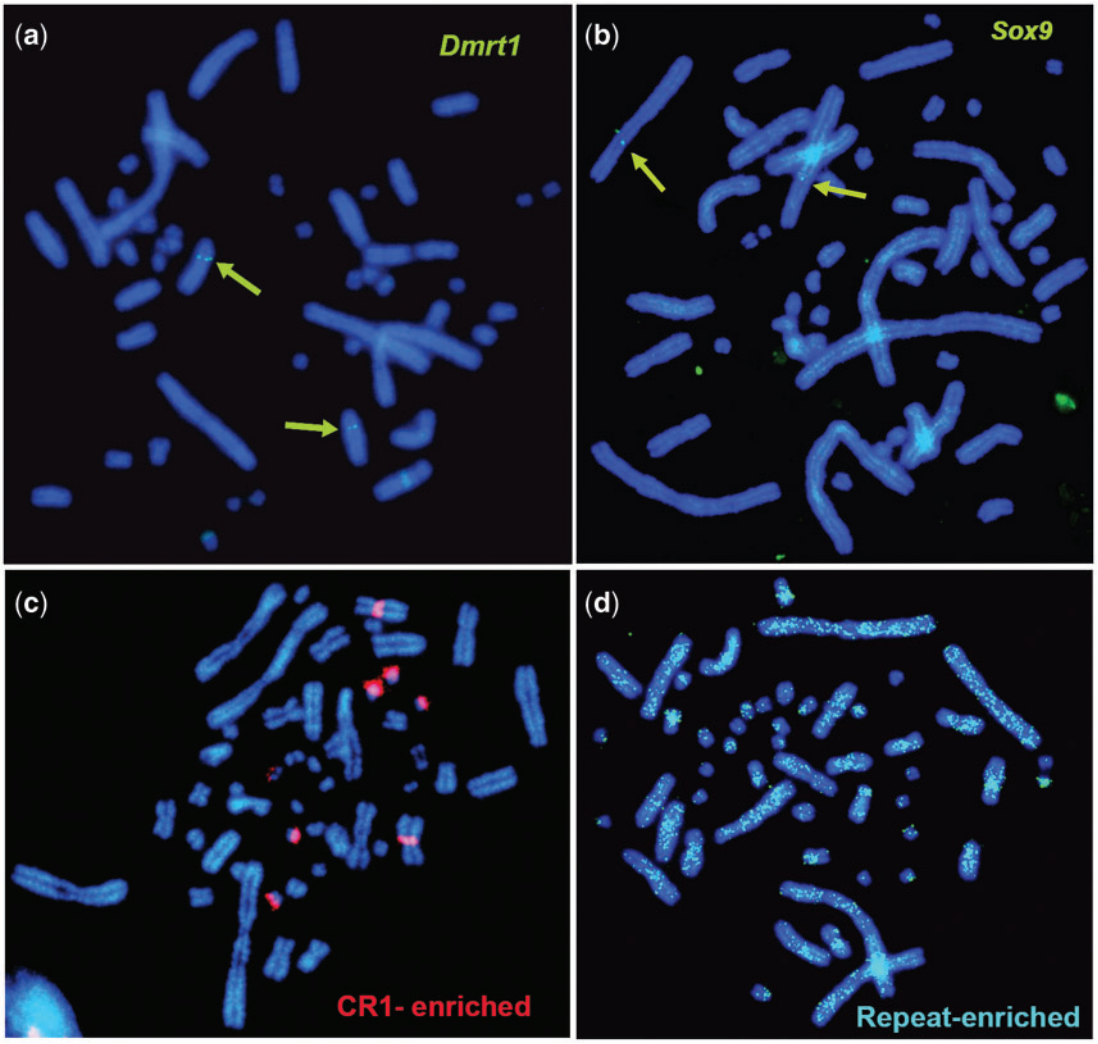

Fig. 3.— Examples of BAC FISH mapping showing the hybridization pattern of BACs containing genes related to sexual development (*a*, *b*), CR1 and Gypsy-enriched BACs (*c*), and simpler repeat-enriched BACs (*d*).

supplementary table S1, Supplementary Material online). Homology to three chicken chromosomes was also detected in CPI-8 and CPI-13, and homology to two chicken chromosomes was identified in CPI-5, CPI-6, CPI-7, and CPI-18. All these CPI chromosomes exhibited homology to between two and five human macrochromosomes. The contrast of gene blocks among species permitted the detection of interchromosomal translocations and inversions in turtle alone, some only in chicken, and others in both turtle and chicken and thus possibly shared across turtles and archosaurs, although tests in crocodilians are needed to confirm this hypothesis (fig. 4).

Our data also revealed syntenic blocks between painted turtle autosomes and amniote sex chromosomes, and the correspondence is not always one to one. For instance, macrochromosomes CPI-7 and CPI-8 harbor gene blocks that are syntenic in CHICKEN-Z, whereas macrochromosomes CPI-1, CPI-9, and microchromosome CPI-22 contain gene blocks orthologous to HUMAN-X (fig. 4). In contrast, CPI-2 contains genes that mapped to snake ELAPHE-Z and ELAPHE-W (table 1). Although available data are scarce for other reptiles, other regions of homology and rearrangements were also detected, involving autosomes and sex chromosomes. Namely, macrochromosome CPI-2 contains a gene block homologous to VARANUS-4, POGONA-6, and ELAPHE-Z (table 1). CPI-2 also shows partial homology to ELAPHE-3, whereas ELAPHE-3 contains another gene block located in CPI-8, and CPI-8 harbors a different gene block that maps to ELAPHE-2, revealing several chromosomal rearrangements between snakes and turtles (table 1). A CPI-4 region appears homologous to PELODISCUS-5 (both macrochromosomes), whereas a gene in CPI-8 (macrochromosome) maps to microchromosome PELODISCUS-13. Some microchromosomes appear to be syntenic across reptiles, as genes in CPI-15 and

BAC-ID	CPI Chr	ARM	CPI Scaffold (3.0.1)	CPI 3.0.1 Start-Stop	CPI Scaffold (3.0.3)	CPI 3.0.3 Start-Stop	GGA Chr	GGA Start-Stop	HSA Chr	HAS Start-Stop	INFERENCES	CHROMOSOME EQUIVALENCY
6L20	1	1p	G-142	22518 - 4149614	n/a	n/a	1	60292896 - 62400801	12	190077 - 1970786	Conserved synteny in reptiles and birds. 6L20 also paints in centromeric region of CPI5 (likely due to the presence of repeats)	CPI1p = GGA1+8+3 = HSA12+22+7+14+20
									22	17084959 - 18149954		
									7	134646779 - 134779413		
68H12			G-12	58178 - 10069935	S-80061	2011284 - 11437061	1	39788270 - 44271369	12	80838126 - 92534054	Interchromosomal insertion in turtles	
							8	3972202 - 4065441	14	105952892 - 105767170		
7H12			Uncharacterized Loci									
67H12			Uncharacterized Loci									
34H12			G-133	95245 - 6587274	n/a	n/a	3	4442435 - 13999212	20	16272097 - 7882984	Conserved synteny in reptiles and mammals; transpositions in birds.	
61H12		1q	S-559	101992 - 2364118	S-80079	82661 - 2270049	1	135634836 - 134393572	2	109335902 - 105654483	Conserved synteny in reptiles and mammals. Scaffold contains Fhl2.	CPI1q = GGA1+5+W = HSA2+X+13+14+19+11
147L13			S-112	1828481 - 4836365	S-87	1579288 - 4234321	1	115540697 - 114932774	X	28587564 - 33339609	Interchromosomal translocation in turtles. BAC contains Dax1	
							7	9821356	2	197804702		
45H12			S-73	5280194 - 1043443	S-80007	23949005 - 20026118	1	162756575 - 165301583	13	58204238 - 41506055	Conserved synteny in reptiles and mammals with inversion in human.	
55A6			S-147	1006242 - 3571919	Chr1	919586 - 3630110	1	177604222 - 178064727	13	21671076 - 20566446	BAC contains Fgf9 Interchromosomal translocation in turtles.	
							5	36250289 - 35798190	14	37197913 - 36298558		
							W	W (NW003766114.1)	19	15468645 - 15237490		
113H12			S-415	106166 - 1890581	S-80008	84632 - 1602563	1	178900223 - 178273314	11	109963673 - 110447759	Interchromosomal insertion in reptiles or translocation in human.	
									13	25456412 - 20397621		
14H12	2	2p	G-68	413259 - 5687491	S-126	354238 - 5083764	2	19615537 - 22659598	7	86974951 - 91875530	Inversion of 2 syntenic blocks in GGA and HAS, & within and between blocks in CPI.	CPI2p = GGA2+22+6insertion = HSA7+10+3+20insertion+8+2+5
									10	91875530 - 15147771		
99H12			S-3	23674 - 11335025	S-80091	6494418 - 17109287	2	32350695 - 39411722	7	26331515 - 30067977	Conserved synteny in birds and turtles. Fussion in reptiles or Fission in HSA.	
									3	15491640 - 28390372		
5H12			G-3	65679 - 10181737	S-39	72243 - 9171490	2	45085043 - 40770095	3	15247733 - 133319449	Virtually conserved synteny in birds and turtles. Inversions in human.	
									7	30323923 - 30536237		
									20	2517253		
25H12			G-122	8998293 - 1991168	S-51	8106476 - 1638554	22	445601 - 2838916	8	35092975 - 42249279	Inversions and intrachromosomal translocation in CPI relative to GGA and HSA. Interchromosomal translocation in turtles.	
							2	69248910 - 71193092	2	74441997 - 95963072		
							6	20550037	5	26880709 - 31400601		
96H12		2q	S-104	148174 - 4402188	S-80083	131352 - 3989799	2	78263832 - 100527616	5	10353751 - 10225619	Virtually conserved synteny in birds and turtles. Single gene insertion in turtles.	CPIq = GGA2+1&4insertion = HSA5+18+7&4insertion+8
									18	7231137 - 3262111		
							1	26299391	7	113876827		
4H12			G-143	111577 - 5002060	S-130	90397 - 4707228	4	81411667	4	3505324	Virtually conserved synteny in birds and turtles. Single gene insertion in turtles.	
							2	114967588 - 112402536	8	67782984 - 61101423		
125H12	3	3p	G-133	95245 - 11266176	S-80062	85958 - 9912304	3	16276727 - 3205835	20	5525080 - 23402156	Inversion in reptiles. Additional inversions in turtles. Independent intrachromosomal translocation in	CPI3p = GGA3 = HSA20
337P6		3q	G-75	237895 - 16082661	S-2	237732 - 13923299	18	4815088 -	17	30782684 - 82273718	Greater syntenic conservation between turtles and mammals,	CPI3q = GGA3+18+27+1insertion = HSA17+6+7insertion
							27	1653359 -				
53H12			S-131	13099 - 4112553	S-181	8142 - 3750497	3	41958947 -	6	166822854 -	Interchromosomal insertion in turtles	
							1	13629485 -	7	104754048 -		
82H12 & 86H12			G-40a	33175 - 17660500	S-80080a	28402 - 16005163	3	63117332 - 53250435	6	31707725	Conserved synteny in reptiles and mammals	
									6	117198376 -		
104H12			S-30	15243 - 6488358	S-80006	13283 - 6113937	3	77046813 - 80212184	6	84262604 - 75794042	Conserved synteny in reptiles and mammals	
31H12			G-37	76118 - 2090886	S-106	76126 - 2086912	3	81940693 - 82664927	6	71566914 - 69345210	Conserved synteny in reptiles and mammals	

Fig. 4.— Chromosomal homology, synteny, and rearrangements identified between CPI turtles and the chicken and human genomes. Multiple gene blocks may be encompassed by the start and stop positions listed within each chromosome and are detailed in the supplementary table S1, Supplementary Material online, along with their gene content.

BAC-ID	CPI Chr	ARM	CPI Scaffold (3.0.1)	CPI 3.0.1 Start-Stop	CPI Scaffold (3.0.3)	CPI 3.0.3 Start-Stop	GGA Chr	GGA Start-Stop	HSA Chr	HAS Start-Stop	INFERENCES	CHROMOSOME EQUIVALENCY
94H12	4	4q	S-572	222859 - 808518	S-465	181972 - 630992	26	2668373 - 2695545	1	208059883 - 208195587	Conserved synteny in reptiles and mammals	CPI4q = GGA26+5+7+1+10 = HSA1+6+11+2+12+2 2+11+15+14
85H12			G-41	156249 - 2148241	S-337	156467 - 2041579	26	1611522 - 1381287	6	36973423 -	Interchromosome translocation in turtles	
									1	111196182 -		
							5	507408 - 489834	11	59341871 -		
88H12			G-125	2138 - 13060113	S-20	96630 - 11623422	7	27985178 -	2	114471354 -	Inter- and intrachromosomal translocation in turtles. Scaffold contains 3'end of Wt1.	
							7	28692690 -	12	40499847 -		
							7	30086800 -	22	46682638 -		
							1	14916473 -	11	28042163 -		
							5	3877671 -	2			
6H12			G-124	278105 - 3023030	S-80086	8838889 - 6346780	5	21105588 - 19802560	11	44282278 - 40135524	Conserved synteny in reptiles and mammals	
36H12			S-152	520559 - 3540026	S-80086	4211749 - 1277139	5	18811456 - 17229662	11	36613493 -	~Conserved synteny in reptiles and mammals	
									11	70313961		
63H12			S-132	49964 - 3817419	S-182	49964 - 3430867	5	29439140 - 28748105	15	33603177 - 49913226	Interchromosomal translocation in turtles and human, plus inversion in human.	
							10	10084599 - 9404625				
106H12			S-75	26303 - 4818454	S-80081	10453847 - 14839738	5	36450791 - 38657380	14	38058757 - 78266426	Conserved synteny in reptiles and mammals, minor inversion in human.	
114H12	5	5p	S-430	101341 - 1641840	S-388	100702 - 1553085	12	4578188 - 5104640	3	11831916 - 128886658	Conserved synteny in reptiles and mammals, minor inversion in human.	CPI5p = GGA12+6 = HSA3+9+10+11
60H12			G-34	31867 - 2200233	S-321	31984 - 2144769	12	4376709 - 3408937	3	11314010 - 10265707	Interchromosomal translocation in reptiles	
									9	34975687 - 35436633		
39B2 & 45D19 & 39D13			G-106	4972 - 3931197	S-82	5135 - 3922778	6	15069667 - 17076431	10	73436805 - 102419222	Inversion in turtles or birds and in mammals. Interchromosomal translocation in mammals.	
									11	7987320		
15H12		5q	S-201	390535 - 3260868	S-80051	1234295 - 3868945	6	31760726 - 30781360	10	126676418 - 124134094	Conserved synteny in reptiles and mammals	CPI5q = GGA6 = HSA10
120H12	6	6a	S-720	78513	S-78603	3096457	4	31564707	4	148078764		CPI6 = GGA4+5 = HSA4+11
33H12			S-549	87402 - 1322407	S-473	84078 - 1218418	4	66760466 - 66247187	4	46037786 - 47596015	Conserved synteny in reptiles and mammals.	
72H12 & 35H18			S-45	285281 - 6283353	S-80080	4450431 - 10208347	4	72818063 -	4	26483018 -	Interchromosomal translocation in turtles	
							5	1107982 -	11	31701317 -		
41L5 & 44L23	7	7a	S-0	365492 - 21830607	Chr7	281849 - 19787469	1	65186102 - 74560219	7	144355341	Greater syntenic conservation between turtles and mammals. Inversions in turtles and birds. Translocations in birds. BAC contains Dmrt1	CPI7 = GGA1+Z+28 = HSA7+12+22+9+18
									12	5432114 -		
									22	42692117 -		
							Z	342471 - 39669740	9	121038 -		
									18	48539056 -		
122H12			G-285	203390 - 6967286	S-80054	156322 - 5614210	Z	3992162 - 7629817	18	39535165 -	Fusion/interchromosomal insertion in reptiles	
									9	31701317 -		
3H12	8	8p	G-278	71360 - 5262819	S-80053	6848731 - 2585669	13	12005652 - 13818438	5	136953189 - 179921412	Inversion and intrachromosomal translocation in HSA.	CPI8p = GGA13 = HSA5
54H12		8q	S-141	41985 - 4022355	S-189	26942 - 3777388	8	7372047 - 5451385	1	179068462 - 165513478	Conserved synteny in reptiles and mammals with inversion in birds	CPI8q = GGA8+Z = HSA1+5
40H12			G-185	16046 - 9762718	S-37	16090 - 9183287	8	3639074 - 9933946	1	192127592 - 179262849	Larger inversion in birds and minor in human.	
38H12			G-248	7913 - 2692049	S-305	7913 - 2416429	8	14916579 - 15670284	1	86889769 - 84335057	Inversion in turtle.	
105H12			S-40	143011 - 6092921	S-95	119705 - 5696689	8	26884366 -	1	64239690 -	Interchromosomal translocation in turtles	
							Z	78579572 -	5	114460459 -		
123H12			S-268	587124 - 3145629	S-80038	505896 - 2670797	8	18513214 - 19322763	1	43610800 - 44679104	Conserved synteny in reptiles and mammals.	
89H12	9	9p	G-145	4018407 - 6824590	S-80073	4166145 - 6779099	4	5935039 - 5117001	X	95939650 - 100168431	Conserved synteny reptiles and mammals.	CPI9p = GGA4 = HSAX
78H12			G-95	17845 - 8537189	S-58	18073 - 7603302	4	1082710 - 9464665	X	68725078 - 86772715	Likely inversions in human and turtles.	
29H12	10	10q	G-180	159163 - 1047325	S-80037	5224269 - 6108070	10	1830470 - 2115125	15	74165922 - 74528630	Inversions in human and turtle.	CPI10q = GGA10 = HSA15
27H12	11	11q	S-587	922 - 1107834	S-80033	2884910 - 3980978	7	10919427 - 10293581	2	202037411 - 200134223	Conserved synteny in reptiles and mammals. Chromosome	CPI11q = GGA7 = HSA2
100H12	13	13p	Uncharacterized Loci									CPI13p = GGA1+16+4 = HSA13+5
26H12			G-123	147516 - 10561036	S-80007	10636193 - 1052452	1	171579052 -	13	37583449 -	Insertions in turtles.	
							16	139264 - 144211	5	180663928 -		
							4	578669	13			
118H12	15	15m	G-168	155584 - 16917475	S-80023	2998366 - 18058274	11	69310 - 10624074	16	68279240 - 46614466	Conserved synteny in reptiles and mammals. Chromosome fusion/translocation in reptiles or fission/translocation in human.	CPI15 (micro) = GGA 11 (micro) = HSA16
									19	30017491 - 34972880		
121H12	18	19m	S-16	225136 - 8825714	S-54	225150 - 7835135	23	3438710 - 5014685	1	41249684 - 3541556	Intrachromosomal translocation and inversion in human. Scaffold contains RSPO1.	CPI19 (micro) = GGA23 (micro) = HSA1
							21	634124 - 989968				

Fig. 4.— Continued.

BAC-ID	CPI Chr	ARM	CPI Scaffold (3.0.1)	CPI 3.0.1 Start-Stop	CPI Scaffold (3.0.3)	CPI 3.0.3 Start-Stop	GGA Chr	GGA Start-Stop	HSA Chr	HAS Start-Stop	INFERENCES	CHROMOSOME EQUIVALENCY
12H12	19	17m	G-225	1837 - 4389059	S-206	1907 - 3508762	3	3037368 - 16091963	6	42896860 - 43149913	Multiple interchromosomal translocations and inversions	CPI17 (micro) = GGA3 (MACRO) = HSA6+20+2
									20	24449835 - 25433333		
									2	38522027 - 113298676		
52H12	21	21 m	S-1038	164879 - 417116	S-681	160148 - 374200	-	-	19	6729925 - 6887560	Conserved synteny in turtles and mammals	CPI21 (micro) = HSA19
116H12	22	22m	S-455	299339 - 1418395	S-403	297272 - 1271452	28	558963	19	2321521	Insertion in turtles	CPI22 (micro) = GGA28 (micro)+1(MA) =
							1	124278003 - 124042084	X	9983795 - 15469064		
28H12	24	24m	G-218	368447 - 14558879	S-17	328830 - 12110944	21	1028530 - 5736965	1	859993 - 20959948	Synteny conserved in reptiles and mammals. Independent inversions and intrachromosomal translocations in turtles, birds and human. Single gene interchromosomal insertion in reptiles.	CPI24 (micro) = GGA21 (micro) = HSA1+16

FIG. 4.— Continued.

Table 1

Partial Homology of Painted Turtle Chromosomes to Those of Other Reptiles

Gene	CPI	VSA	LRE	EQU	PSI	PVI	SCR	STR	Reference
EPB41L3	2			3p					1
TOP2B	2	4q							2
TAX1BP1	2			Zp		6p			1, 3
RAB5A	2			Zp, Wq		6p			1, 3
CTNNB1	2	4q	6q	Zp, Zcen, Wcen		6p			1, 2, 3
KAT2B	2					6p			3
ARG1	3				3q				4
WT1	4	2q	1q	1q			X, Y		2, 5
COQ6	4				5q				4
EIF2B2	4				5q				4
CTBP2	5					3q			3
DMRT1	7							X, Y	6
DCTN4	8			2q					1
RUFY1	8	1q	2q	2q					2
TPR	8			3p					1
RPE65	8	8p							2
SPARC	8				Micro 13				4
BRD7	15	Micro	Micro	Micro					2
ENO1	24	Micro		Micro					2

NOTE.—VSA, *Varanus salvator macromaculatus*; LRE, *Leiolepsis reevesii rubritaeniata*; EQU, *Elaphe quadrivirgata*; PSI, *Pelodiscus sinensis*; PVI, *Pogona vitticeps*; SCR, *Siebenrockiella crassicollis*; STR, *Staurotypus triporcatus*; cen, centromeric; Micro, microchromosome. References: 1, Matsubara et al. (2006); 2, Srikulnath et al. (2013); 3, Young et al. (2013); 4, Matsuda et al. (2005); 5, Kawagoshi et al. (2012); 6, Kawagoshi et al. (2014). Shaded cells denote a split region in turtle with respect to snakes.

CPI-24 map to VARANUS, LEIOLEPIS, and ELAPHE microchromosomes as well (table 1).

Of the 61 BACs that mapped successfully to a single location, seven contained genes or mapped to scaffolds containing genes in the sex determination network of turtles and vertebrates (*FgF9*, *Dax1*, *Sox9, Dmrt1, Fhl2, Wt1,* and *Rspo1*) (Valenzuela 2008a; Badenhorst et al. 2013). The relative position of these genes revealed additional chromosomal rearrangements among amniotes and regions of homology between CPI autosomes and sex chromosomes in other taxa. Namely, BACs containing *Fhl2*, *FgF9* and *Dax1* mapped to CPI-1q and to CHICKEN-1, whereas this gene block is split in human as they map to chromosome HUMAN-2 (Chan et al. 1998), HUMAN-13 (Mattei et al. 1995), and HUMAN-X (Zanaria et al. 1994), respectively. Additionally, *Sox9* mapped to CPI-3q whereas it is located on the CHICKEN-18 microchromosome (Kuroiwa et al. 2002) and on HUMAN-17 (Foster et al. 1994). G-banding (fig. 2) and whole-chromosome-specific painting using *Trachemys scripta* (TRACHEMYS) probes indicate that CPI-3 is orthologous to TRACHEMYS-3 (Badenhorst D,

Montiel Jiménez EE, Stanyon R, Ferguson-Smith MA, O'Brien PCM, Valenzuela N, unpublished data), which in turn appears homologous to CHICKEN-3 (Kasai et al. 2012) (i.e., CPI-3 = TRACHEMYS-3 = CHICKEN-3). Therefore, our results suggest the transposition of *Sox9* chromosomal location between macrochromosomes in turtles (*Sox9* = CPI-3 = TRACHEMYS-3) and a chicken microchromosome (*Sox9* = CHICKEN-18). On the other hand, *Dmrt1* mapped to CPI-7 (our study, fig. 3*a*), and it is located in *Gekko hokouensis* lizards GEKKO-*Z* (Kawai et al. 2009), and in PELODISCUS-6 which is homologous to CHICKEN-Z (Kawai et al. 2007). CHICKEN-Z in turn is homologous to *Staurotypus triporcatus* turtles STAUROTYPUS-X/Y (Kawagoshi et al. 2014). As our chromosome-specific painting shows CPI-7 to be homologous to TRACHEMYS-6 (Badenhorst D, Montiel Jiménez EE, Stanyon R, Ferguson-Smith MA, O'Brien PCM, Valenzuela N, unpublished data), then CPI-7 appears to be homologous to PELODISCUS-6 as well. The apparent conserved autosomal synteny of *Dmrt1* (a strong candidate for avian sex-determining gene [Smith et al. 2009]) across turtles is of interest, because CPI exhibits TSD and lacks sex chromosomes (Valenzuela et al. 2014), whereas *P. sinensis* displays a ZZ/ZW sex-determining system (Kawai et al. 2007), and PELODISCUS-Z is homologous to CHICKEN-15 (Kawagoshi et al. 2009). Furthermore, *Wt1*, a candidate gene for a role as a TSD master gene in CPI based on transcriptional profiling (Valenzuela 2008b; Valenzuela et al. 2013), maps to CPI-4 and this region shows homology to CHICKEN-5 and HUMAN-11, and *Siebenrockiella crassicollis* turtles SIEBENROCKIELLA-X/Y (Kawagoshi et al. 2012). These observations combined with the homology of CPI-7 to CHICKEN-Z and GEKKO-Z, and of CPI-2 to ELAPHE-Z and POGONA-6 (table 1) of *Po. vitticeps*, a lizard with ZZ/ZW micro sex chromosomes (Ezaz et al. 2005), all support the notion that the mechanisms of sex-determination have evolved independently between birds and turtles (Kawai et al. 2007), as well as among turtles, snakes, and lizards (Ezaz et al. 2009). Otherwise, all reptilian sex chromosomes would have shown homology to a single CPI chromosome.

Conclusion

In summary, our study extends the currently available cytogenetic and DNA sequence (Shaffer et al. 2013) data for painted turtles, an emerging model for ecology and evolution (Valenzuela 2009). Importantly, the improved assembly and physical mapping presented here advance our understanding of the evolution of amniote genomes. For instance, our data reveal that macrochromosome synteny is not fully retained between birds and turtles for the six largest chromosomes as previously reported between turtles and archosaurs (birds and crocodilians) (Matsuda et al. 2005; Kasai et al. 2012). Indeed, rearrangements were identified involving both these and other macro and microchromosomes. Our results also support the notion that sex-determining mechanisms have evolved independently multiple times in birds, turtles, and squamates. Indeed, regions in seven different CPI chromosomes show homology to sex chromosomes of other turtles, birds, squamates, and human, supporting the idea that not one but multiple chromosomes were recruited as sex chromosomes in different vertebrate lineages. We hope that this study, the first of its kind in turtles and TSD vertebrates, fosters further research into the fascinating evolution of vertebrate genomes.

Supplementary Material

Supplementary information and tables S1–S3 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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