Cytogenetic Insights into the Evolution of Chromosomes and Sex Determination Reveal Striking Homology of Turtle Sex Chromosomes to Amphibian Autosomes

Eugenia E. Montiel\textsuperscript{a} Daleen Badenhorst\textsuperscript{a} Ling S. Lee\textsuperscript{a} Robert Literman\textsuperscript{a} Vladimir Trifonov\textsuperscript{b} Nicole Valenzuela\textsuperscript{a}

\textsuperscript{a}Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, Iowa, USA; \textsuperscript{b}Institute of Molecular and Cellular Biology SB RAS, Novosibirsk, Russian Federation

\section*{Abstract}

Turtle karyotypes are highly conserved compared to other vertebrates; yet, variation in diploid number (2n = 26–68) reflects profound genomic reorganization, which correlates with evolutionary turnovers in sex determination. We evaluated the published literature and newly collected comparative cytogenetic data (G- and C-banding, 18S-NOR, and telomere-FISH mapping) from 13 species spanning 2n = 28–68 to revisit turtle genome evolution and sex determination. Interstitial telomeric sites were detected in multiple lineages that underwent diploid number and sex determination turnovers, suggesting chromosomal rearrangements. C-banding revealed potential interspecific variation in centromere composition and interstitial heterochromatin at secondary constrictions. 18S-NORs were detected in secondary constrictions in a single chromosomal pair per species, refuting previous reports of multiple NORs in turtles. 18S-NORs are linked to ZW chromosomes in \textit{Apalone} and \textit{Pelodiscus} and to X (not Y) in \textit{Staurotypus}. Notably, comparative genomics across amniotes revealed that the sex chromosomes of several turtles, as well as mammals and some lizards, are homologous to components of \textit{Xenopus tropicalis} XTR1 (carrying \textit{Dmrt1}). Other turtle sex chromosomes are homologous to XTR4 (carrying \textit{Wt1}). Interestingly, all known turtle sex chromosomes, except in Trionychidae, evolved via inversions around \textit{Dmrt1} or \textit{Wt1}. Thus, XTR1 appears to represent an amniote proto-sex chromosome (perhaps linked ancestrally to XTR4) that gave rise to turtle and other amniote sex chromosomes.

\section*{Key Words}

18S rDNA · C-bands · Evolution · Homology · Interstitial telomeres · Karyotype evolution · NOR · Reptile · Sex chromosome · Sex determination · Turtle

The compartmentalization of genomes into chromosomes alters genome function, because the regulatory environment of genes is influenced by their synteny or lack thereof [Ahituv et al., 2005], and co-expressed genes tend to be clustered in the genome [de Wit and van Steensel, 2009]. Thus, discerning the causes and consequences of genome compartmentalization is key to understanding genome function, because chromosome fusion or fission events alter the number of chromosomes and the synteny of the genes they contain. Reptilian genomes vary in their size, organization, and composition.
Fig. 1. Ancestral reconstruction of diploid chromosome numbers for turtle species with known diploid numbers and sex determination mechanisms. Evolutionary transitions between temperature-dependent sex determination (TSD) and genotypic sex determination (GSD) are indicated in the branches where they were reconstructed to have occurred by maximum likelihood [Valenzuela and Adams, 2011]. GSD = GSD with uncharacterized sex chromosome system. TSDIa = TSD where males are produced at lower and females at higher temperature. TSDII = TSD where males are produced at intermediate temperatures and females at lower and higher values. Three-letter acronyms are shown for the 13 target species whose cytogenetic data were collected during this study. Branch color denotes ancestral state reconstruction by parsimony following Valenzuela and Adams [2011], whereas numbers at key nodes denote reconstruction by maximum likelihood using the ace function of ape version 3.4 [Popescu et al., 2012] in R [R Core Development Team, 2012].
[Olmo, 2008]. Genome evolution has received increased attention as new genomic resources are developed for reptiles, a group that holds an important key to understanding vertebrate evolution [Wang et al., 2006; Janes et al., 2008; Alfoldi et al., 2011; Castoe et al., 2013; Shaffer et al., 2013; Vonk et al., 2013; Wang et al., 2013; Green et al., 2014].

Within reptiles, turtles have been considered to possess highly conserved karyotypes [Olmo, 2008; Pokorná et al., 2011] as some chromosomes appear to be unchanged for over 200 million years [Bickham, 1981]. Yet, the wide variation in chromosome number across turtles (fig. 1) point to drastic changes in genome organization that took place during this time, and these rearrangements are curiously associated with evolutionary transitions in sex-determining mechanisms [Valenzuela and Adams, 2011]. Here, we provide new cytogenetic data from selected species on the evolution of turtle karyotypes and review our knowledge of chromosomal sex determination in turtles.

Fig. 2. G-banded karyotypes of 13 target species examined in this study and their phylogenetic relationships. Taxonomic families are indicated at the corresponding nodes and sex-determining mechanisms are color coded. Green arrowheads denote the chromosomal location of the NORs.
Methods

Cell Culture, Chromosome Preparation, and Chromosome Banding

Chromosome preparations were obtained from fibroblast cell cultures established from embryonic or muscle tissue following standard procedures as previously described [Valenzuela, 2009; Badenhorst et al., 2013, 2015; Valenzuela et al., 2014; Montiel et al., 2016]. New samples were obtained from private collections or pet trade specimens. 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 CO₂ supplementation [Badenhorst et al., 2013]. Four hours prior to harvesting, 10 μg/ml colcemid (KaryoMAX®, Invitrogen) was added to the cultures, which were harvested after 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].

NOR Mapping by 18S rRNA FISH

The distribution of NORs was investigated by FISH using a turtle-specific 18S DNA fragment labeled by nick-translation and co-precipitated with salmon sperm DNA [Badenhorst et al., 2013].

Telomere FISH Mapping

A telomeric probe containing the repeat motif (TTAGGG)n was constructed and labelled with biotin or DIG-dUTP by PCR following Ijdo et al. [1991]. The probe was hybridized to metaphase chromosomes of all target species following Badenhorst et al. [2013]. Briefly, chromosome spreads and the DNA probe were phase chromosomes of all target species following Badenhorst et al. [2013]. The probe was hybridized to metaphase chromosomes of all target species following Badenhorst et al. [2013].

Results and Discussion

Evolution of Turtle Karyotypes

Here, we present newly collected comparative cytogenetic data (G- and C-banding, 18S rRNA FISH mapping (18S-NOR), and telomere FISH mapping) for 13 turtle species spanning the full spectrum of diploid numbers (2n = 28–68) except for the lowest 2n = 26 (figs. 1, 2). We refer to these species by their genus names or 3-letter acronyms hereafter. A summary of new and previously published data is presented in table 1. While standardized systems of chromosome nomenclature exist for some organisms, including humans, domestic animals, and some plants [e.g., Linde-Laursen et al., 1997; Cribiu et al., 2001; Simons et al., 2013], no such system exists for turtles. This is partly due to the scarcity of uniform banding information across taxa, which limits our understanding of turtle genome macroevolution [Olmo, 2005]. Here, we adopted the organization of chromosomes by decreasing size and centromere position as is commonly used today. Namely, G-banded karyotypes (fig. 2) were ordered by approximate size. In general, smaller microchromosomes were nearly indistinguishable from each other by shape and centromere position, and thus, they were ordered by approximate size and G-banding pattern where possible.

The diploid number in turtles ranges from 2n = 26 in Peltocephalus dumeriliani [Killebrew, 1975a; Ventura et al., 2014] to 2n = 68 in Carettochelys insculpta [Bickham et al., 1983] with an average of 2n = 52 given our current knowledge of turtle chromosomes [Olmo and Signorino, 2005] (fig. 1). Our comparative cytogenetic data (fig. 2) illustrate a general characteristic of turtle karyotypes that is shared by birds and squamates, namely, the presence of 2 distinct components, macro- and microchromosomes [Olmo et al., 2002]. The presence of microchromosomes appears to be the ancestral condition to amniotes as it is also found in ancestral fish and amphibian lineages, and they appear to have fused among themselves in crocodilians leading to their absence in this group [Uno et al., 2012]. Our data also underscore that most variation in turtle diploid number is due to differences in the number of microchromosomes (fig. 2).

Our cytogenetic data confirmed previously reported diploid numbers for all species examined (fig. 2, table 1). However, while the numbers of macro- and microchromosomes were in agreement with previous reports for Trachemys (TSC), Chrysemys (CPI), Glyptemys (GIN), and Emydura (EMA) (all 26:24), Pelodiscus (PSI) (16:50), and Apalone (ASP) (18:48) (table 1), they differed in Sternotherus (SOD) (22:34) from previous reports (26:30 reported by Killebrew [1975b]), Staurotypus (STR) (22:32) (26:28 reported by Killebrew [1975b]), Cheylida (CSE) (22:30) (28:24 reported by De Smet [1978]), Chelodina (COB) (24:30) (22:32 reported by Bull and Legler [1980]), Carettochelys (CIN) (18:50) (16:52 reported by Bickham et al. [1983]), Pelomedusa (PSU) (24:12) (26:10 reported by De Smet [1978]), and Podocnemis (PUN) (22:6) (10:18 reported by Olmo and Signorino [2005]). These discrepancies in the number of macro-
### Table 1. Summary of cytogenetic results from 13 target turtles examined in this study

<table>
<thead>
<tr>
<th>Suborder</th>
<th>Family or superfAMILY</th>
<th>Species</th>
<th>Acronym</th>
<th>Diploid number</th>
<th>Macro-chromosomes</th>
<th>Micro-chromosomes</th>
<th>18S FISH NOR</th>
<th>Chromosome pairs with ITS</th>
<th>Confirmed reports</th>
<th>Countered reports</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptodira</td>
<td>Emydidae</td>
<td><em>Chrysemys picta</em></td>
<td>CPI</td>
<td>50</td>
<td>26</td>
<td>24</td>
<td>14</td>
<td>none</td>
<td>Killebrew [1977]; Badenhorst et al. [2015]</td>
<td>De Smet [1978]</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Glyptemys insculpta</em></td>
<td>GIN</td>
<td>50</td>
<td>26</td>
<td>24</td>
<td>14</td>
<td>9</td>
<td>Bickham [1975]; Montiel et al. [2016]</td>
<td></td>
</tr>
<tr>
<td>Chelydridae</td>
<td></td>
<td><em>Chelydra serpentina</em></td>
<td>CSE</td>
<td>52</td>
<td>22</td>
<td>30</td>
<td>4</td>
<td>1, 2, 3, 5, 8, 9, 11</td>
<td>Stock [1972]</td>
<td></td>
</tr>
<tr>
<td>Kinosternidae</td>
<td></td>
<td><em>Staurotypus triporcatus</em></td>
<td>STR</td>
<td>54</td>
<td>22</td>
<td>32</td>
<td>X</td>
<td>1, 2</td>
<td>Sites et al. [1979] (S. salvini); Bickham and Rogers [1985] (S. salvini)</td>
<td>Killebrew [1975a]; Kawagoshi et al. [2014]</td>
</tr>
<tr>
<td>Trionychidae</td>
<td></td>
<td><em>Pelodiscus sinensis</em></td>
<td>PSI</td>
<td>66</td>
<td>16</td>
<td>50</td>
<td>ZW</td>
<td>none</td>
<td>Sato and Ota [2001]; Kawai et al. [2007]</td>
<td>Matsuda et al. [2005]; Kawai et al. [2007]</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Apteronotus spinifer</em></td>
<td>ASP</td>
<td>66</td>
<td>18</td>
<td>48</td>
<td>ZW</td>
<td>none</td>
<td>Sato and Ota [2001]; Badenhorst et al. [2013]</td>
<td>Killebrew et al. [1983]; Bickham et al. [1983]</td>
</tr>
<tr>
<td>Carettochelyidae</td>
<td><em>Carettochelys insculpta</em></td>
<td>CIN</td>
<td>68</td>
<td>18</td>
<td>50</td>
<td>2</td>
<td>none</td>
<td>Bickham [1976]</td>
<td>Bull and Legler [1980]; Martinez et al. [2008]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Chelodina oblonga</em></td>
<td>COB</td>
<td>54</td>
<td>24</td>
<td>30</td>
<td>micro</td>
<td>none</td>
<td>Bull and Legler [1980]</td>
<td></td>
</tr>
</tbody>
</table>

**Bold font = Novel data not published before; underlined font = data counter to published reports; plain font = data matching published reports.**
microchromosomes reflect differences in the ability to identify chromosomal homologs for the smaller chromosome pairs during various studies, and also differences in the classification criteria employed by researchers. This is due to the difficulty presented when chromosomes are less elongated in the metaphase spread preparations, which renders some smaller chromosomes harder to distinguish from microchromosomes.

Ancestral reconstruction of chromosome number by parsimony, maximum likelihood (fig. 1), or using Bayesian models [Organ et al., 2008] identified 2 drastic evolutionary changes in each of the 2 turtle suborders. Namely, a radical reduction in diploid number within the side-neck suborder Pleurodira appears to have taken place in Pelomedusoidea (Podocnemididae + Pelomedusidae), whereas within the hidden-neck suborder Cryptodira a dramatic increase in chromosome number took place in the family Trionychidae (fig. 1). In Pleurodira, Pelomedusoidea has low diploid numbers and few microchromosomes (2n = 26–36), whereas Chelidae is characterized by higher diploid numbers and the presence of numerous microchromosomes (2n = 50–58), a characteristic similar to the karyotypes found in Cryptodira [Bull and Legler, 1980] (fig. 2).

The distribution of heterochromatin exhibits variation among chelonians. The C-positive material corresponding to constitutive heterochromatin is largely restricted to the centromeric regions in almost all chromosomes in all species examined except in Pelomedusoidea (fig. 3; online suppl. fig. 1, for all online suppl. material, see www.karger.com/doi/10.1159/000447478). The variation in centromere C-banding (some centromeres are strongly C-positive, other only faintly, and few are C-negative) may reflect true differences in the composition of turtle centromeric sequences as occurs in other organisms [Steiner and Henikoff, 2015], perhaps even differences in their origin. Further research is needed to uncover compositional differences among turtle centromeres that might exist to test this hypothesis. In addition, some microchromosomes showed a heterochromatic p arm in all emydids. Furthermore, interstitial C-positive heterochromatin exists in the p arm of chromosomes GIN9, GINY, SOD5, CSE6, the q arm of PSI5, and some microchromosomes of Sternotherus, Stuatrotypus, Chelydra, and Pelodiscus (fig. 3; online suppl. fig. 1). Distal C-positive signals were detected in the q arm of SOD1, STR1, COB4, COB6, and in the telomere region of PSI1, PSI3, and ASP1 (online suppl. fig. 1G). COB4 was heteromorphic in size and G-banding of the p arm (the largest homolog of the pair exhibits an extra distal G-band on the p arm). Interestingly, C-banding results in a more intense interstitial band than the centromeric bands in Pelomedusoidea. Interstitial heterochromatin is more limited in Pelomedusa (PSU1 and PSU5), while in Podocnemis it encompasses 6 chromosome pairs (PUN1–PUN5 and PUN8; fig. 3E, F). In all these cases in Podocnemis, this interstitial heterochromatin corresponds with secondary constrictions (1 in PUN3, PUN5, and PUN8 and 2 in PUN1, PUN2, and PUN4). Interstitial heterochromatin is also visible in one microchromosome pair in Trachemyx, Chrysemys, Glyptemys, Sternotherus, and one macrochromosome pair in Carettochelys (CIN2; fig. 3D), Stuatrotypus (STRX), and one homolog of the NOR pair
in *Emydura* (EMA14; online suppl. fig. 1F). This interstitial C-positive material corresponds to the NOR in *Chrysemys* [Badenhorst et al., 2015], as well as *Sternotherus, Staurotypus*, and *Emydura* [this study], *Podocnemis* (one of the multiple sites only), and to the NOR-bearing region in *Trachemys, Glyptemys, Carettochelys*, as detailed below. None of the interstitial C bands correspond to the NOR in *Pelomedusa*.

**Turtle NOR Evolution**

The NOR contains the genes for the 18S, 5.8S, and 28S ribosomal RNAs [Shaw and McKeown, 2011]. NORs are frequently located on a secondary constriction of chromosomes but not always [Bickham and Rogers, 1985; Prieto and McStay, 2008], and this condition has been proposed as a conserved trait in turtles [Cleiton and Giuliano-Caetano, 2008]. Turtle NORs have been investigated in a number of turtles, mostly using the secondary constriction method (scNOR) [e.g., Huang and Clark, 1969; De Smet, 1978] or silver staining (AgNOR) [e.g., Bickham and Rogers, 1985; Martinez et al., 2009; Fantin and dos Santos Monjelo, 2011; Badenhorst et al., 2015], which exposes the active NOR by dyeing the proteins associated with rDNA activity [Howell, 1977]. However, not all secondary constrictions correspond to NORs such that this method can be misleading [Bickham and Rogers, 1985]. Also, because genomes may retain inactive copies of the NOR after translocations have occurred, silver staining reveals only a partial view of NOR’s evolutionary history. Thus, we also used 18S rDNA FISH (18S-NOR) as previously applied to a few turtle taxa [Kawai et al., 2007; Cleiton and Giuliano-Caetano, 2008; Badenhorst et al., 2013, 2015; Kawagoshi et al., 2014] to detect potentially inactive NOR copies that may have passed undetected in some of the turtles previously examined using AgNOR or scNOR techniques. Our 18S FISH data of the 13 target taxa includes the first reports of 18S-NORs for 7 of them (GIN, CSE, SOD, CIN, EMA, COB, and PSU) (table 1).

Our combined results and published data indicate that all turtles thus far studied possess a unique NOR located on a single chromosome pair (fig. 4; online suppl. fig. 2). This is the most common condition in reptiles [Olmo and Signorino, 2005], amphibians [King et al., 1990; Schmid and Steinlein, 2015], and fish [Gornung, 2013], although some species in these lineages possess multiple NORs, as do humans, other mammals [Prieto and McStay, 2008; Farley et al., 2015], and birds [de Oliveira Barbosa et al., 2013]. Importantly, existing data indicate that previous reports of multiple NORs in turtles are in error, likely because they were based on the secondary constriction method. Namely, 3 NOR-bearing chromosomes were identified in *Trachemys dorbigni* and 2 in *Batagur trivittata* based on secondary constrictions [De Smet, 1978], but later AgNOR and 18S FISH studies demonstrated that the *T. dorbigni* NOR is located on a single chromosome [Cleiton and Giuliano-Caetano, 2008] as in *T. scripta* studied here (fig. 4, table 1; online suppl. fig. 2). Similarly, 7 NOR-bearing chromosomes were reported in *Podocnemis expansa* and *P. unifilis* based on secondary constrictions [Huang and Clark, 1969], but our data indicate the existence of a single NOR in PUN1 (fig. 4, table 1). Thus, the reports for *P. expansa* and *B. trivittata* based on the assumption that all secondary constrictions are indicative...
of NORs may also be mistaken. Therefore, current data support the notion that the turtle NOR has changed its chromosomal location among taxa, and yet, no inactive copies of NORs exist in any of the examined chelonians as revealed by 18S FISH (fig. 4; online suppl. fig. 2). Thus, when the NOR moved to a new chromosome from its ancestral location in some turtle lineages, it did so by a complete translocation without evidence in any chromosome of a NOR duplication event having occurred.

**Turtle Telomere Evolution**

As expected, our telomeric (TTAGGG)n probe [Ijdo et al., 1991] hybridized to the termini of all the chromosomes in the species investigated (fig. 5; online suppl. fig. 3). This observation is consistent with reports that telomeres are crucial for maintaining chromosomal stability as they protect chromosome ends during cell division, repair of double-strand breaks, and ensure the viability of chromosomes products from fission events [Flint et al., 1994; de Lange, 2002; Bolzan and Bianchi, 2006; Gomes et al., 2010]. However, non-terminal TTAGGG signals were detected (interstitial telomeric sites, ITS) in several of our target species, including the centromeric region of 7 chromosome pairs in *Chelydra* (CSE1, 2, 3, 5, 8, 9, 11) and *Podocnemis* (PUN1, 2, 3, 4, 5, 6, 7), 2 pairs in *Staurotypus* (STR1, 2), and 1 pair in *Glyptemys* (GIN9) (fig. 5, table 1; online suppl. fig. 3). The ITS in *Podocnemis* do not co-localize with the secondary constrictions where C-positive interstitial heterochromatin was detected (fig. 5F). ITS are commonly found in fish [Ocalewicz, 2013], birds [Nanda et al., 2002], humans [Azzalin et al., 2001], and other mammals [Finato et al., 2000; Ruiz-Herrera et al., 2005, 2008; Castiglia et al., 2007], and unlike terminal telomeres, these repetitive regions can be linked to chromosomal instability and rearrangements that may lead to genome reorganization and speciation [Ishii et al., 2008; Brown and O’Neill, 2010]. Indeed, telomeric repeats correlate with chromosomal evolutionary breakpoints [Flint et al., 1994; Azzalin et al., 2001; Ruiz-Herrera et al., 2002, 2005, 2008].

The observation that changes in chromosome number occurred in the turtle lineages where ITS were found (fig. 1; CSE 2n = from 54 to 52, PUN 2n = from 38 to 28, GIN 2n = from 52 to 50, STR 2n = from 56 to 54) suggests that some of these turtle ITS perhaps derive from chromosomal fusions [Azzalin et al., 2001] which will decrease chromosome number. But whether these turtle ITS constitute such ghosts-of-fusions-past requires further testing, as fusion ITS may be characterized by stretches of tandem telomeric repeats that are oriented head-to-head [Azzalin et al., 2001]. A different explanation is that these sites were inserted during the repair of double-strand breaks [Azzalin et al., 2001] and thus represent the relics of evolutionary genome reorganization events other than fusions [Ruiz-Herrera et al., 2005]. Also, telomeric sequences are sometimes found interspersed in the rRNA gene cluster, which may also represent fragile sites prone to fission [Rousselet et al., 2000] that would increase chromosome number. However, this alternative can be ruled out in our target taxa, since no ITS co-localized with the NORs in any of the species examined here. Finally, telomeres appear to be the evolutionary precursors of eukaryote centromeres [Villasante et al., 2007; Mendez-Lago et al., 2009;
Garavis et al., 2013], and telomeric DNA may give rise to neocentromeres [Villasante et al., 2007; Ishii et al., 2008] as reported in plants, invertebrates, and mammals [Fukagawa and Earnshaw, 2014; Steiner and Henikoff, 2015]. But whether some of the observed turtle ITS represent evolutionary neocentromeres remains to be tested.

**Evolution of Turtle Sex Determination**

Of the 335 recognized turtle species [van Dijk et al., 2014], 87 have a known sex-determining mechanism (SDM) established based on either incubation experiments or cytogenetic evidence [Tree of Sex Consortium, 2014; Montiel et al., 2016]. Phylogenetic analyses recover temperature-dependent sex determination (TSD) as the ancestral SDM in turtles, and most evolutionary transitions involved the evolution of genotypic sex determination (GSD) [Janzen and Krenz, 2004; Valenzuela and Adams, 2011], with 2 potential reversals back to TSD inferred by maximum likelihood analysis [Valenzuela and Adams, 2011] (fig. 1). Of the 18 turtles known to possess GSD, 10 have a cytogenetically characterized system of heterogamety (figs. 1, 6), 3 in the suborder Pleurodira which are all XX/XY [McBee et al., 1985; Martinez et al., 2008; Ezaz et al., 2009], and 7 in Cryptodira, 3 of which are ZZ/ZW [Sharma et al., 1975; Kawai et al., 2007; Badenhorst et al., 2013] and 4 are XX/XY [Bull et al., 1974; Carr and Bickham, 1981; Montiel et al., 2016]. Of these 10 cases, 3 involved microchromosomes and 7 macrochromosomes. Relatively little is known about the molecular content of turtle sex chromosomes, although information is increasing [Kawagoshi et al., 2009, 2012, 2014; Montiel et al. 2016]. Molecular cytogenetic data from comparative genome hybridization (CGH) has been used to identify the extent of the male- or female-limited region of the turtle sex chromosomes [Ezaz et al., 2006; Kawai et al., 2007; Martinez et al., 2008; Badenhorst et al., 2013; Montiel et al., 2016] where recombination should be reduced or absent. These data suggest that the pseudoautosomal region (segments of sex chromosomes that do recombine in XY males or ZW females [Bachtrog et al., 2011]) is miniscule in Chelodina longicollis [Ezaz et al., 2006] which possesses micro sex chromosomes, potentially larger in

---

**Fig. 6.** Homology of some turtle sex chromosome systems to chicken, some lizards, human, and *Xenopus tropicalis*. Arrows denote chromosomal inversions. *X. tropicalis* homology to chicken is based on Brelsford et al. [2013] and Warren et al. [2010]. CPI = *Chrysemys picta*, GIN = *Glyptemys insculpta*, SCR = *Siebenrockiella crassicollis*, STR = *Staurotypus triporcatus*, PSI = *Pelodiscus sinensis*, ASP = *Apalone spinifera*, GGA = *Gallus gallus*, GHO = *Gekko hokouensis*, ACA = *Anolis carolinensis*, TSE = *Takydromus sexlineatus*, HSA = *Homo sapiens*, XTR = *Xenopus tropicalis*, *Wt1* = Wilm’s tumor suppressor gene 1, *Dmrt1* = doublesex and Mab-3 related transcription factor 1.
Turtle sex chromosomes may be the carriers of the NOR (Staurotypus [Sites et al., 1979; Bickham and Rogers, 1985]; Pelodiscus [Kawai et al., 2007], this study, fig. 4; Apalone [Badenhorst et al., 2013], this study, fig. 4), as it also occurs in diverse taxa [reviewed in Literman et al., 2014] such as some reptiles, mammals, amphibians, and fish [Goodpasture and Bloom, 1975; Hsu et al., 1975; Schmid et al., 1983, 1993; Porter et al., 1994; Born and Bertollo, 2000; Wiley, 2003; Abramyan et al., 2009; Takehana et al., 2012], as well as some invertebrates [e.g., Monti et al., 2011; Montiel et al., 2012]. The NOR in ASPW and PSIW is much larger than the Z-NOR in these turtles, a dimorphic trait that can be leveraged to diagnose the genotypic sex in these turtles using molecular techniques [Literman et al., 2014]. In contrast, we found that in STR the 18S-NOR is located on the X chromosomes exclusively and not on the Y, as previously described in the congener Staurotypus salvinii [Sites et al., 1979; Bickham and Rogers, 1985]. Loss of a NOR by a deletion in one homolog of the chromosomal pair harboring them may explain the evolutionary origin of the lack of Y-NORs observed in STR, as a single NOR was detected in one individual examined of the TSD turtle Kinosternon subrubrum and in 1 out of 4 individuals studied of the XX/XY turtle Siebenrockiella crassicollis whose NORs are autosomal [Bickham and Rogers, 1985]. Alternatively, the lack of a NOR in STRY may be the result of the degeneration of the Y in STR as part of the evolutionary dynamics of sex chromosomes [Bachtrog et al., 2011], or the result of the translocation of the NOR to the X chromosome exclusively (and not to the Y) as occurred in Hyla femoralis [Wiley, 2003]. However, X- and Y-linked NORs were detected in S. triporcatus and S. salvinii by Kawagoshi et al. [2014] using a combined 18S-28S probe, suggesting that instead, the STRY retains 28S rRNA genes but lost 18S rRNA genes. Further research is needed to test these alternatives.

Recent studies showed that chromosome 1 of Xenopus tropicalis (XTR1) is homologous to the sex chromosomes in 3 divergent anurans and birds [Brelsford et al., 2013], and existing data indicate that this deep homology extends to turtles as well, as it does to other amniotes including lizards and humans (fig. 6). Namely, XTR1 contains genomic blocks homologous to zebra finch (TGU) chromosomes TGU4, TGU15, TGU26, TGU28, and TGUZ, and zebra finch in turn shows high homology with chicken (GGA) chromosomes [Warren et al., 2010] such that XTR1 contains genomic blocks homologous to GGA4, GGA15, GGA26, GGA28, and GGAZ. Notably, comparative genomics revealed that the XY chromosomes of Staurotypus are homologous to GGAZ [Kawagoshi et al., 2014] as are the lizard Gekko hokouensis ZW [Kawai et al., 2009]. The ZW of Pelodiscus and Apalone are homologous to GGA15 [Badenhorst et al., 2013; Kawagoshi et al., 2009] as is the Anolis X [Gamble et al., 2014; Rovatsos et al., 2014], and the p arm of Glyptemys GINX/Y maps to GGA26 [Badenhorst et al., 2015; Montiel et al., 2016]. Moreover, the block of XTR1 that is homologous to GGA4 is in turn homologous to human X and the lizard Takydromus sexlineatus X [Rovatsos et al., 2016], while the XTR1 block homologous to GGA28 and CIP22 maps to the human X [Badenhorst et al., 2015] (fig. 6). On the other hand, most of GINXY and Siebenrockiella SCRXY (which evolved independently from each other) exhibit homology with GGA5 [Montiel et al., 2016] and XTR4. Thus, these observations reveal that the sex chromosomes of Staurotypus, Pelodiscus, Apalone, and Glyptemys (in part) are homologous to XTR1, while the sex chromosomes of Siebenrockiella and most of Glyptemys are homologous to XTR4 (fig. 6). Interestingly, XTR1 carries Dmrt1, a gene co-opted for a sex-determining function in birds and various other vertebrates and whose molecular evolution is linked to evolutionary turnovers of sex determination in reptiles [Janes et al., 2014]. Further, XTR4 carries Wt1, another important regulator of gonadal formation and putative master TSD gene in Chrysemys [Valenzuela, 2008]. Moreover, the evolution of turtle sex chromosome systems in all these cases involved chromosomal inversions, and in all but Trionychid turtles, these inversions encompassed Dmrt1 or Wt1 [Kawagoshi et al., 2009, 2012, 2014; Montiel et al., 2016]. The evolutionary origin of the Pangshura smithii ZZ/ZW system [Sharma et al., 1975] in the same turtle family Geoemydidae as Siebenrockiella, as well as the origin of the sex chromosomes of chelid turtles (Emydura, Chelodina, and Acanthochelys radiolata), remains unknown.

Thus, current data support the hypothesis that multiple turtle chromosomes have been co-opted as sex chromosomes (and sometimes repeatedly) during the independent evolution of GSD in various lineages [Montiel et al., 2016], and it appears that this process may have been facilitated by their particular gene content, which seems to make them good at determining sex [O’Meally et al., 2012]. Furthermore, the proclivity of these genomic regions to take on a role as sex chromosomes and master
activators of the sex determination cascade seem to emanate from their deep shared homology to what appears to be a proto sex chromosome in non-amniotic tetrapods.

Acknowledgements

We thank J. Iversion, B. McCord, G. Rivera, as well as C. Innis and New England Aquarium for kindly donating samples, R. Stanyon for his generous advice on lab procedures, and J. Iversion for editorial comments. This work was funded in part by NSF grants MCB 0815354 and MCB 1244355 to N.V.

References


Statement of Ethics

All procedures were approved by the IACUC of Iowa State University.

Disclosure Statement

The authors have no conflicts of interest to declare.
Turtle Sex Chromosome Homology to Amphibian Autosomes

Cytogenet Genome Res 2016;148:292–304
DOI: 10.1159/000447478


Montiel/Badenhorst/Lee/Literman/Trifonov/Valenzuela