

A ZZ/ZW microchromosome system in the spiny softshell turtle, *Apalone spinifera*, reveals an intriguing sex chromosome conservation in Trionychidae

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Abstract Reptiles display a wide diversity of sex-determining mechanisms ranging from temperature-dependent sex determination (TSD) to genotypic sex determination (GSD) with either male (XY) or female (ZW) heterogamety. Despite this astounding variability, the origin, structure, and evolution of sex chromosomes remain poorly understood. In turtles, TSD is purportedly ancestral while GSD arose multiple times independently. Here we test whether independent (XY or ZW) or morphologically divergent heterogametic sex chromosome systems evolved in tryonichids (Cryptodira) using the GSD spiny softshell turtle, *Apalone spinifera*, a species with previously unidentified sex chromosomes. A female-specific signal from comparative genomic hybridization (CGH) was detected in a Giemsa/4',6-diamidino-2-phenylindole faint portion of a microchromosome, indicating the presence of

a ZZ/ZW system in *A. spinifera*. In situ hybridization of a fluorescently labeled 18S rRNA probe identified a large nucleolar organizer region block in the female-specific region of the W (co-localizing with the female-specific CGH signal) and a smaller block on the Z. The heteromorphic ZZ/ZW microsex chromosome system detected here is identical to that found in another tryonichid, the Chinese softshell turtle *Pelodiscus sinensis*, from which *A. spinifera* diverged ~95 million years ago. These results reveal a striking sex chromosome conservation in tryonichids, compared to the divergent sex chromosome morphology observed among younger XX/XY systems in pleurodiran turtles. Our findings highlight the need to understand the drivers behind sex chromosome lability and conservation in different lineages and contribute to our knowledge of sex chromosome evolution in reptiles and vertebrates.

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Abbreviations

2n	Diploid number
CGH	Comparative genomic hybridization
DAPI	4',6-diamidino-2-phenylindole
FISH	Fluorescent in situ hybridization
gDNA	Genomic DNA
GSD	Genotypic sex determination

mya	Million years ago
my	Million years
NOR	Nucleolar organizer region
rRNA	Ribosomal RNA
TSD	Temperature-dependent sex determination
XX/XY	Male heterogamety
ZZ/ZW	Female heterogamety

Introduction

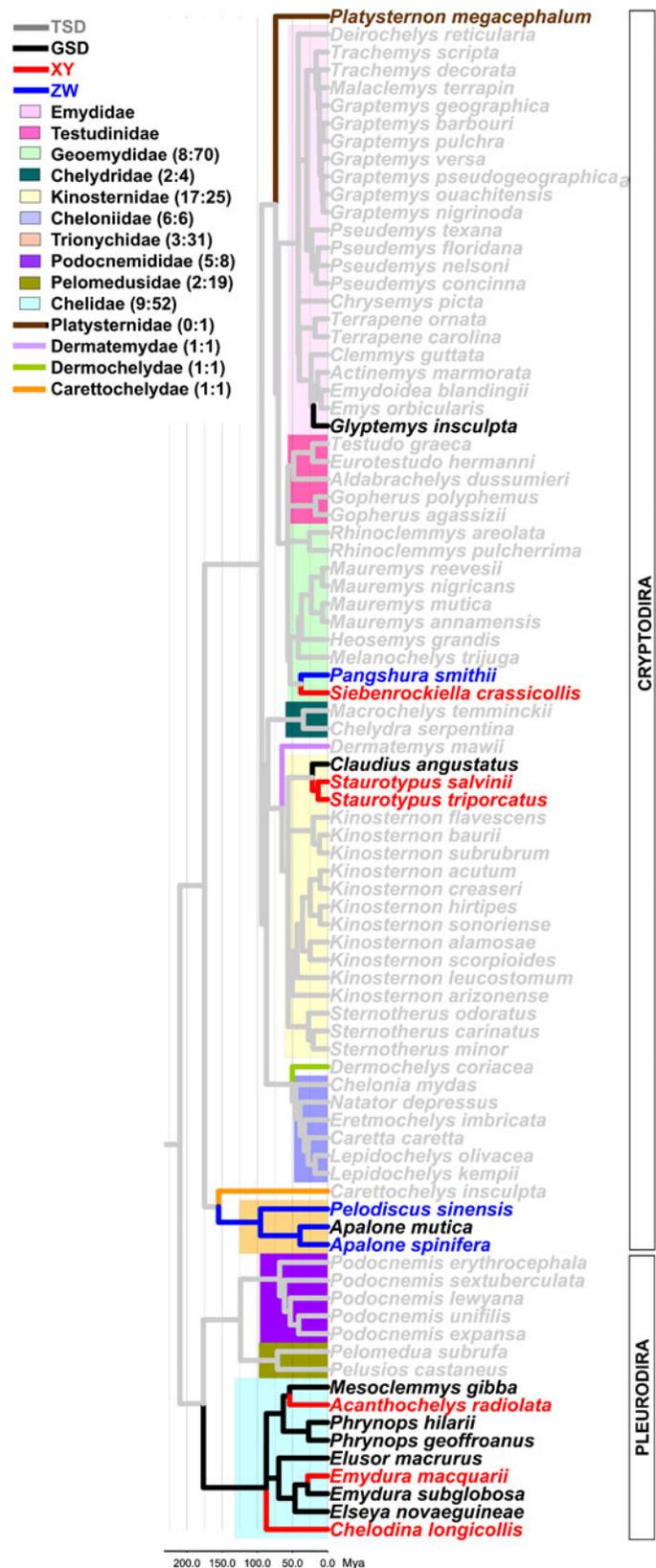
Sexually reproducing organisms employ an astonishing diversity of mechanisms to produce males and females, spanning systems under environmental control (environmental sex determination) to systems under genotypic control (genotypic sex determination, GSD) (Bull 1983; Solari 1994; Valenzuela et al. 2003; Valenzuela 2004). The degree of evolutionary conservation of sex-determining mechanisms varies as well, as demonstrated among vertebrate lineages (Bull 1983; Solari 1994; Valenzuela 2004). For instance, all birds exhibit female heterogametic (ZZ/ZW) and most mammals exhibit male heterogametic (XX/XY) sex chromosome systems, while reptiles and fish vary from temperature-dependent sex determination (TSD) to GSD with either XX/XY or ZZ/ZW systems (Solari 1994; Scherer and Schmid 2001; Valenzuela and Lance 2004). Sex chromosomes are known to play an important role in the evolution of sex ratio, sexual selection, sexual dimorphism, and sexual conflict in GSD taxa (Bachtrog et al. 2011). However, our understanding of the evolution of sex chromosomes and of the traits and processes they influence is hindered by the paucity of data, both on the origin and prevalence of sex chromosomes across species, as is the case in turtles.

Turtles exhibit both TSD and GSD (ZZ/ZW and XX/XY) sex-determining systems and encompass a diverse range of chromosome complements ($2N=28-66$; Valenzuela and Adams 2011). Although TSD is more prevalent in turtles than GSD (Fig. 1), the identification of GSD in this clade was more frequently inferred from the minimal effect of temperature on sex ratios (Greenbaum and Carr 2001) than documented by cytogenetic analysis. Differentiated sex chromosomes were only detected in eight out of the approximately 20 turtle species known to possess GSD (Valenzuela 2004); (Bull et al. 1974; Sharma et al. 1975; Carr and Bickham 1981; McBee et al. 1985;

Ezaz et al. 2006; Kawai et al. 2007; Martinez et al. 2008). Current phylogenetic analyses posit that GSD is a derived trait in turtles (Janzen and Krenz 2004) that evolved once in the suborder Pleurodira in the family Chelidae and multiple times independently in the suborder Cryptodira in the families Emydidae, Geoemydidae, Kinosternidae, and Trionychidae (Fig. 1). The XX/XY sex chromosome system identified in four chelid turtles could be explained by the single origin of GSD when this family split from its sister TSD lineage (Pelomedusidae + Podocnemididae; Fig. 1). In contrast to Pleurodira, multiple origins of GSD in Cryptodira are reflected by (or consistent with) the coexistence of ZZ/ZW and XX/XY systems within a single family (e.g., Geoemydidae; Fig. 1). Despite the apparent single origin of GSD in Chelidae, the morphology of its XY chromosomes has diverged significantly among species, contrasting with placental mammals where the X chromosome is largely conserved across disparate taxa (Graves 2006). For example, *Chelodina longicollis* has a micro-sex chromosome system (Ezaz et al. 2006), while both *Emydura macquarii* and *Acanthochelys radiolata* have macro-sex chromosomes (McBee et al. 1985; Martinez et al. 2008). Furthermore, although *E. macquarii* and *A. radiolata* share the same type of sex chromosome system, the morphology of the Y differs between them; large vs. degenerate Y chromosome, respectively (McBee et al. 1985; Martinez et al. 2008).

In the suborder Pleurodira, all studied softshell turtles in the family Trionychidae have GSD, and a ZZ/ZW system was recently reported in the Chinese softshell turtle, *Pelodiscus sinensis* (Kawai et al. 2007), which may reflect a single origin of ZZ/ZW at the base of this lineage (Fig. 1). However, given that male and female heterogametic sex chromosome systems are present within Geoemydidae, namely, XX/XY in *Siebenrockiella crassicollis* (Carr and Bickham 1981) and ZZ/ZW in *Pangshura smithii* (Sharma et al. 1975), it is possible that contrasting heterogametic systems may have also evolved independently in Trionychidae. Alternatively, an ancestral ZZ/ZW system may have diverged morphologically in Trionychidae as seen in Chelidae (McBee et al. 1985; Ezaz et al. 2006; Martinez et al. 2008). *Apalone* and *Pelodiscus* softshell turtles diverged ~95 million years ago (mya; Fig. 1), while *Chelodina* and *Emydura* are separated by ~90 my (Fig. 1). Thus, it is possible that sufficient time has elapsed to permit similar morphological evolution to occur in Trionychidae as seen in Chelidae.

Fig. 1 Pruned phylogenetic tree showing the phylogenetic relationships among all turtle species with known sex-determining mechanisms. Families with GSD are highlighted in color. Numbers in parenthesis separated by a colon indicate the species with known sex-determining mechanism (left) and the total number of species (right) per family. Topology and data from Valenzuela and Lance (2004), Iverson et al. (2007), and Valenzuela and Adams (2011)



Here, we test these alternative hypotheses, specifically whether different heterogametic sex chromosome systems (XY vs. ZW) or a morphologically divergent ZZ/ZW arose within Trionychidae. We do so by characterizing the sex chromosome system of another species in this family, the spiny softshell turtle *Apalone spinifera*. Previous cytogenetic studies using conventional cytogenetic techniques such as G-banding failed to identify heteromorphic sex chromosomes in *A. spinifera* (Vogt and Bull 1982; Bickham et al. 1983; Greenbaum and Carr 2001), potentially due to the presence of homomorphic sex chromosomes (similar size and morphology of macro or microchromosome pair) or of cryptic heteromorphic sex chromosomes that require high-resolution cytogenetic techniques for their identification. One such technique, comparative genomic hybridization (CGH), has enabled the detection of cryptic sex chromosomes in several reptiles, i.e., the turtles *C. longicollis* (Ezaz et al. 2006), *E. macquarii* (Martinez et al. 2008), *P. sinensis* (Kawai et al. 2007), and the lizard *Pogona vitticeps* (Ezaz et al. 2005). Here, we report on the application of G-banding, C-banding, and CGH to identify the sex chromosomes of *A. spinifera*. We include the trionychid turtle *P. sinensis* in our analyses from a different sampling site from that reported by Kawai et al. (2007) to enable direct interspecific comparisons. Additionally, we carried out fluorescent in situ hybridization (FISH) with 18S ribosomal RNA (rRNA) to map the location of the nucleolar organizer regions (NORs) as the NORs localized in the Z and W chromosomes in *P. sinensis* (Kawai et al. 2007).

Materials and methods

Animal and tissue sampling

Four adult female and two adult male *A. spinifera* were obtained from a turtle farm in Iowa and their sex was determined by examination of sexually dimorphic external morphology. Tissue from one adult male and one female *P. sinensis* was collected from an introduced, wild population on Oahu, Hawaii, and stored in collection media (Ezaz et al. 2008) until processing. Tissue biopsies were collected from the tail of each animal and used for cell culture. All procedures were approved by the IACUC of Iowa State University.

Cell culture, chromosome preparation, and G- and C-bands

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. Four hours prior to harvesting, 10 µg/ml colcemid (Roche) was added to the cultures. Metaphase chromosomes were harvested and fixed in 3:1 methanol/acetic acid. Cell suspensions were dropped onto glass slides and air dried. The application of G- and C-banding to *A. spinifera* chromosome spreads followed conventional protocols (Seabright 1971; Sumner 1972). A minimum of ten complete metaphases were analyzed for each specimen. Images were captured with a CCD camera coupled to an Olympus BX41 fluorescence microscope and analyzed using Genus Imaging Software (Applied Imaging) and karyotypes were ordered in decreasing size.

18S rRNA FISH mapping

In order to isolate the 18S rRNA gene, the following primers were designed from available 18S rRNA sequence data for *Trachemys scripta* (GenBank accession no. M59398.1): 5' GACCCTGTAATTGGAATGAGTAC 3' (forward) and 5' GTTCATTATCGGAATTAACCAGAC 3' (reverse). The 18S rRNA was amplified from genomic DNA (gDNA) by PCR in a 25-µl reaction containing 20–100 ng gDNA, 2.5 µl Taq buffer (10×), 0.75 µl MgCl₂ (50 mM), 1.25 µl dNTPS (2.5 mM), 0.5 µl of each forward and reverse primer (10 µM) and 0.5 µl Taq (5 U/µl), and dH₂O. Cycling parameters entailed an initial denaturing step of 94 °C for 3 min followed by 25 cycles of: 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 90 s and a final extension of 72 °C for 10 min. PCR products were labeled by nick translation with digoxigenin-dUTP (Roche), and 18S rRNA FISH was carried out by hybridizing labeled probes to *A. spinifera* and *P. sinensis* chromosome preparations. Sealed chromosome spreads with probe were denatured together on a hot plate at 65 °C for 3 min. Hybridization took place in a humid chamber at 37 °C for two nights. Post-hybridization washes consisted of a first wash in 0.4× SSC/0.3 % Tween 20 for 2 min at

60 °C, followed by second wash in 2× SSC/0.1 % Tween 20 for 1 min at room temperature. The fluorescence detection solution comprised 4XT/relevant antibody in a 250- μ l final volume at 37 °C for 20–45 min. Slides were subsequently washed thrice in 4XT at 37 °C, counterstained with 4',6-diamidino-2-phenylindole (DAPI) (6 μ l 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.

Comparative genomic hybridization

Male and female gDNA of *A. spinifera* was labeled with digoxigenin-dUTP or biotin-dUTP using a nick translation kit (Roche) following the manufacturer's instructions. CGH was performed as described by Ezaz et al. (2005). Briefly, chromosome slides were hardened at 65 °C for 2 h, denatured for 2 min at 70 °C in 70 % formamide/2× SSC, and dehydrated through an ethanol series and air dried. For each slide, a 15- μ l mixture containing 250–500 ng of digoxigenin-dUTP female and biotin-dUTP male was co-precipitated with 5–10 μ g boiled gDNA from the homogametic sex (as competitor) and 20 μ l glycogen (as carrier) and was hybridized to one slide at 37 °C for 3 days. As G-band results indicated that male *A. spinifera* was the homogametic sex, only male gDNA was used as a competitor for *A. spinifera* chromosome preparations. The detection protocol was carried out as previously described for 18S rRNA FISH (see above). Each sex-by-color combination was tested in reciprocal experiments and a minimum of ten complete metaphases were analyzed for each specimen. An identical procedure was applied to *P. sinensis*.

Results

A. spinifera karyotype

G-banded mitotic karyotypes of four female and two male *A. spinifera* were analyzed. The karyotypes obtained from females (Fig. 2a) and males (Fig. 2b) were virtually identical and confirmed the diploid number of 2n=66 previously reported for *A. spinifera* (Stock

1972; Bickham et al. 1983). Nine pairs of macrochromosomes and 24 pairs of microchromosomes were identified, differing slightly from the report by Bickham et al. (1983) of eight pairs of macrochromosomes. The macrochromosomes identified here included two pairs of metacentric, four pairs of submetacentric, and three pairs of acrocentric chromosomes (Fig. 2). The centromere position of the 24 pairs of microchromosomes could not be detected accurately due to their small size, which impedes the unambiguous pairing of some microchromosomes with their G-banded homologue.

Comparison between the four female and two male karyotypes revealed a morphologically heteromorphic chromosome pair in females. This female-specific chromosome was a relatively large microchromosome containing a large Giemsa faint block (weakly stained by Giemsa; Fig. 2a). C-band analysis supports these findings as a large C-positive block was identified on a heteromorphic microchromosome, which is absent in the male *A. spinifera* karyotype (Fig. 2c–e). The C-positive block corresponds with the Giemsa faint region of the heteromorphic chromosome.

18S rRNA FISH mapping

The hybridization signals from 18S rRNA FISH localized to two microchromosomes in both female (Fig. 3a, d) and male *A. spinifera* (Fig. 3b, d). A similar pattern was observed in female (Fig. 3c) and male *P. sinensis* (data not shown), corroborating Kawai et al. (2007) findings in a Japanese *P. sinensis* population. Similar to *P. sinensis* (Fig. 3c; Kawai et al. 2007), a distinct difference was observed in the size of the signal between the two microchromosomes in the four female *A. spinifera*, where the hybridization signal on one homologue was much larger than on the other, reflecting a potential difference in gene copy number. No such difference was observed in the two males. The chromosome where the larger 18S rRNA hybridization signal was observed in females corresponded with the heteromorphic chromosome identified using G- and C-banding and the signal localized at the Giemsa faint region (see Fig. 2). DAPI staining was also faint in this region, indicating that it is GC-rich. The size of the 18S rRNA signal was much smaller on the other female microchromosome homologue (Fig. 3a, d), and it appears homologous with the microchromosome pair bearing the 18S rRNA gene in males (Fig. 3b, d).

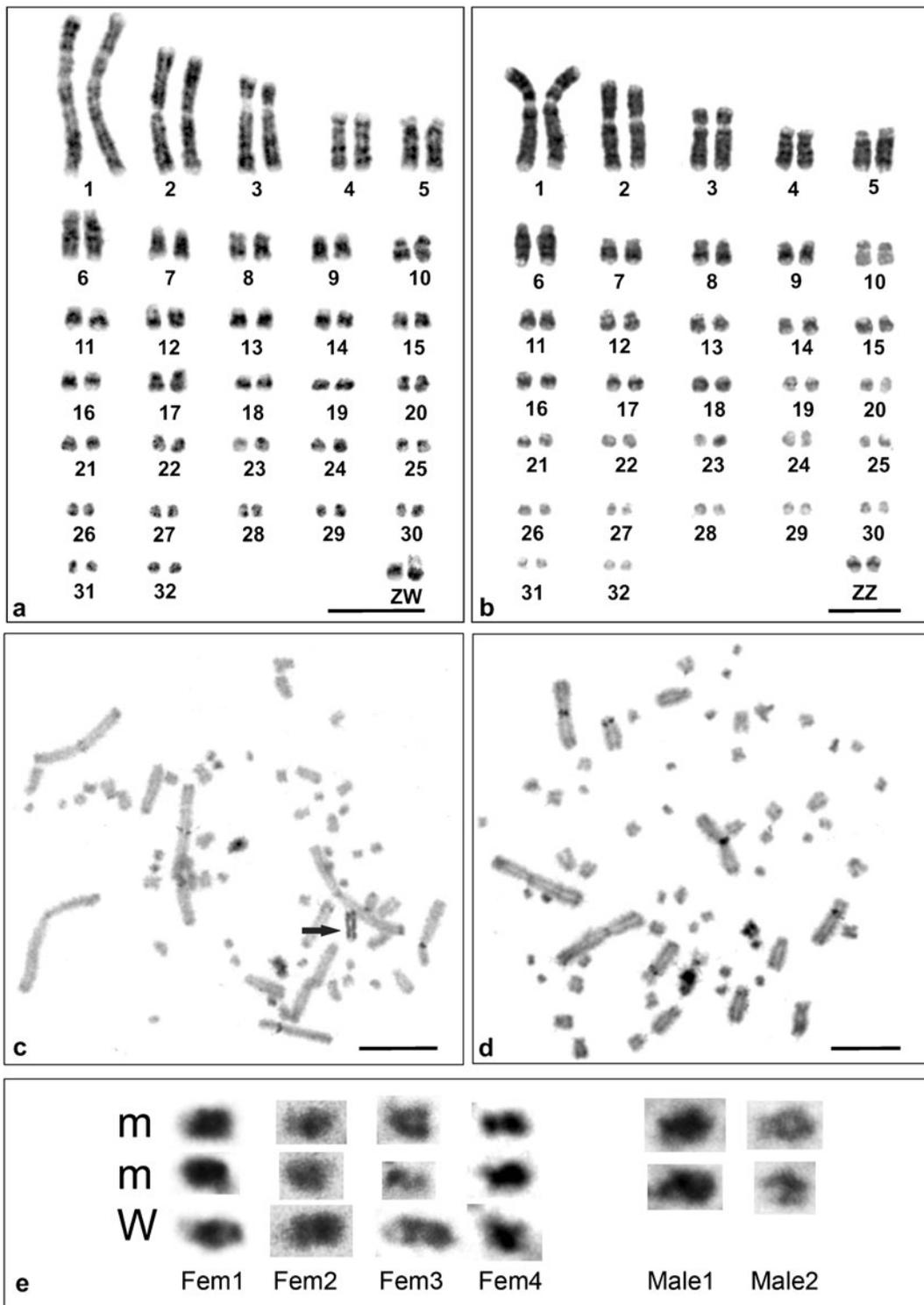


Fig. 2 G- and C-banded metaphase chromosome karyotypes of female (**a**, **c**) and male (**b**, **d**) *A. spinifera*, respectively. **e** Enlarged images of the highly heterochromatic microchromosomes in *A. spinifera*, indicating the W in female (*Fem*) and the heterochromatic microchromosome pair (*m*) in both females and males. A large

block of the female-specific chromosome (*W*) is Giemsa faint and C-positive. The *arrow* indicates the female-specific chromosomes with large C-positive block. Note that the Z is morphologically indistinguishable from several other microchromosomes with similar banding pattern. Scale bar=10 μ m

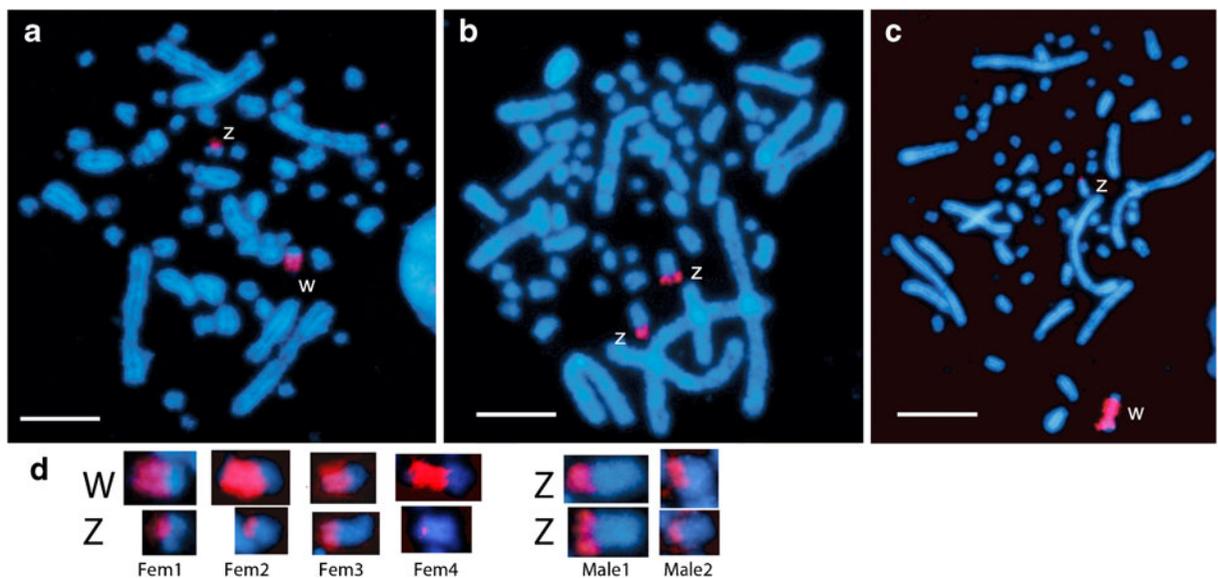


Fig. 3 Chromosomal distribution of the 18S rRNA gene on metaphase chromosome spreads in female (**a**) and male (**b**) *A. spinifera*. 18S rRNA FISH signals are located on a pair of heteromorphic

chromosomes in female (*Fem*; **a**, **d**) and a pair of the same-sized microchromosomes in male (**b**, **d**). **c** 18S rRNA FISH distribution in female *P. sinensis* chromosome spreads. Scale bar=10 μ m

Comparative genomic hybridization

The CGH of differentially labeled and co-precipitated female and male gDNA produced a sex-specific pattern in *A. spinifera* (Fig. 4). A differential hybridization signal was identified in the four females involving the female-specific heteromorphic chromosome (Fig. 4a), whereas no differential signal was detected in the two male *A. spinifera* spreads (Fig. 4d). This CGH female-specific region co-localized with the Giemsa/DAPI-faint block where the larger 18S rRNA hybridization signal was also detected on the heteromorphic female-specific microchromosome (W) (Figs. 2 and 3). Taken together, all data are consistent with the existence of a ZZ/ZW sex chromosome system in *A. spinifera* comprising a larger W and a smaller Z microchromosome. Identical results were obtained for *P. sinensis* (Fig. 4).

Discussion

Understanding the patterns and processes underlying the evolution of sex determination remains an area of active research (Valenzuela 2010; Bachtrog et al. 2011); one that relies on the correct identification of sex chromosomes and accurate characterization of sex-determining mechanisms (Valenzuela et al. 2003). Theoretical

models predict that sex chromosome evolution generally leads to the degeneration of the heterogametic sex chromosome (Y or W) (Charlesworth et al. 2005), a process that may have significant fitness costs due to, for example, the loss of genes from the heterogametic chromosome (Graves 2004; Charlesworth et al. 2005). However, seemingly homomorphic sex chromosomes have been identified in ancient lineages (Pigozzi and Solari 1999; Nakamura 2009) and may represent an alternative evolutionarily stable state (Valenzuela 2010). This is of particular interest in reptiles where sex-determining systems are highly labile and span both genetic and environmental sex-determining systems, as well as male and female heterogamety that differ in their level of heteromorphism.

Here we examined the karyotype of the GSD turtle *A. spinifera* and our G-banding and CGH permitted the identification of distinguishable heteromorphic sex chromosomes involving a pair of microchromosomes that were undetected in previous cytogenetic studies (Stock 1972; Bickham et al. 1983). Our data revealed that *A. spinifera* possesses a ZZ/ZW-type sex-determining system and represents the third report of female heterogamety in turtles after *P. smithii* (Sharma et al. 1975) and *P. sinensis* (Kawai et al. 2007). This is also the third report of micro-sex chromosomes in turtles (Ezaz et al. 2006; Kawai et al. 2007), which along with the occurrence of micro-sex chromosomes

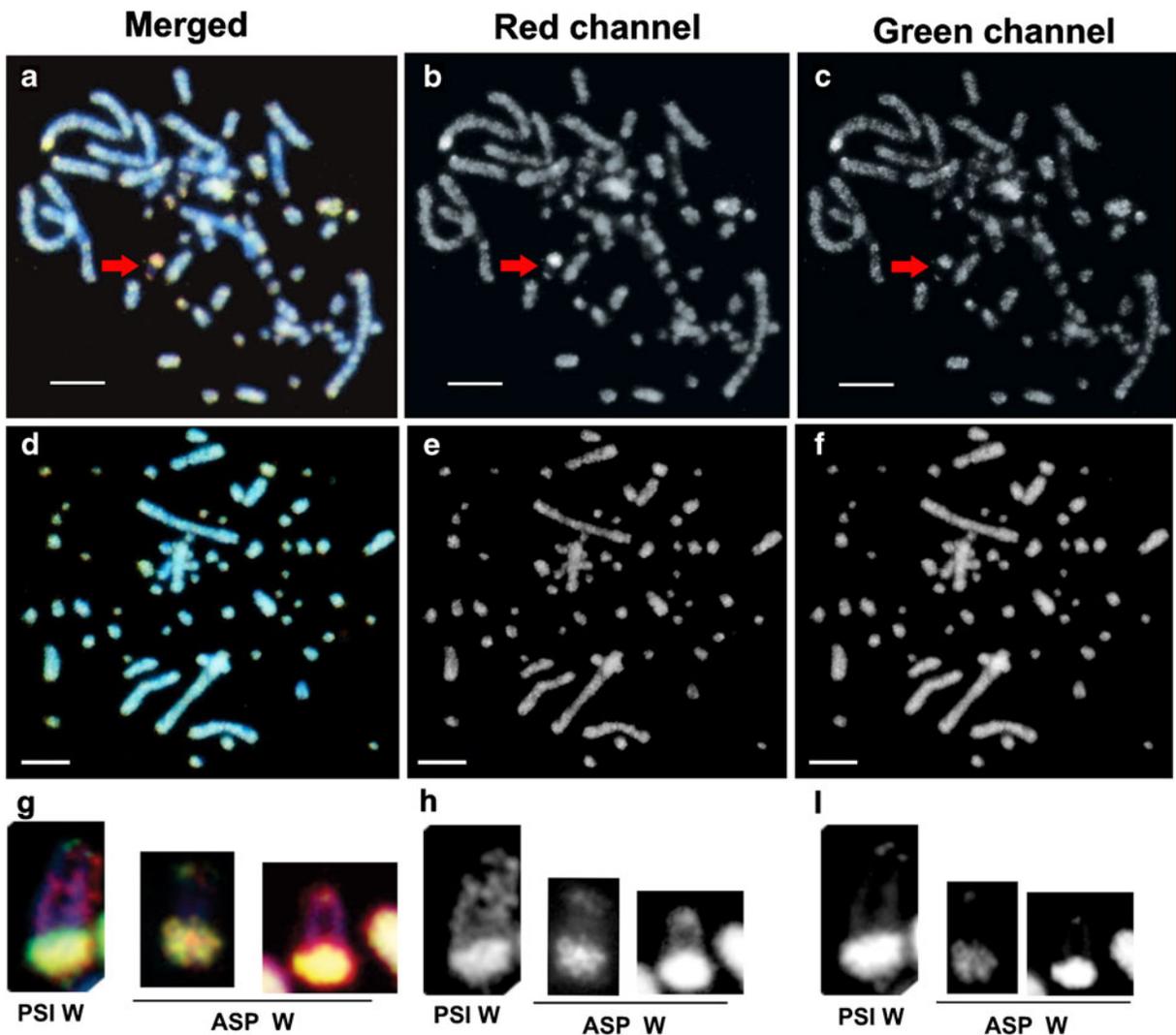


Fig. 4 CGH on female (a–c) and male (d–f) *A. spinifera* chromosome spreads and (g–i) enlarged images of CGH on female *P. sinensis* and *A. spinifera* W chromosomes. a, d, g Merged CGH image. b, e, h Digoxigenin-dUTP-labeled female total genomic

DNA. c, f, i Biotin-dUTP-labeled male total genomic DNA. Arrows indicate the *A. spinifera* W chromosome. Note the lack of any differential signal in males. Scale bar=10 μ m

in several species of lizards (Ezaz et al. 2005, 2009, 2010) supports the suggestion that micro-sex chromosomes may be a more prevalent system in GSD reptiles than previously anticipated. Additionally, the 18S rRNA FISH signal mapped to the sex chromosomes in *A. spinifera* and revealed a large nucleolar organizer region (NOR) block on the female-specific micro chromosome (W) and a smaller block on its counterpart (Z; Fig. 3). This NOR signal co-localizes with the CGH female-specific signal and is non-overlapping with the DAPI-strong region of the W. This larger NOR block was observed in the W of all four females

while the smaller 18S signal was found in the Z of the two males and all females (Fig. 3). Thus, the differential size of the NOR between males and females contributes greatly to the dimorphism between the larger W and smaller Z in this species. Our results, including the large C-positive block on the W (Fig. 2c), concur with reports that Y or W chromosomes generally accumulate repetitive sequences (Lepesant et al. 2012) and indicate that the 18S rRNA in *A. spinifera* and *P. sinensis* is interspersed with C-positive heterochromatin repeats. These findings are important as there is an increasing number of reports of NORs on sex chromosomes (albeit

cases remain infrequent), with those of *A. spinifera* and *P. sinensis* representing the first reptilian cases. Other examples include fish (Moran et al. 1996; Reed and Phillips 1997; Born and Bertollo 2000; Takehana et al. 2012), frogs (Schmid et al. 1983, 1993; Green 1988; Wiley 2003; Abramyan et al. 2009), rat kangaroo and fruit bats (Goodpasture and Bloom 1975), orangutan (Schempp et al. 1993), and white-cheeked gibbon (Supanum et al. 2012).

The location of NORs on the Z and W is significant as they may be subjected to the same evolutionary forces that act on sex chromosome evolution (Bachtrog et al. 2011; Otto et al. 2011). For instance, the reduction of recombination that typically follows the establishment of the sex-determining region facilitates the accumulation of heterochromatin, decreasing recombination even further (Bergero and Charlesworth 2009). The reduction or suppression of recombination may allow different combinations of alleles to evolve independently in the homogametic and heterogametic sex chromosomes, enabling adaptations by the protection of favorable allele combinations (Ayala and Coluzzi 2005; Kirkpatrick and Barton 2006). Additionally, the accumulation of heterochromatin on the *A. spinifera* W may have significant functional impacts, as the closer a gene is to a heterochromatic block, the greater the chance that its expression may be altered (Kleinjan and van Heyningen 1998). In this regard, the dimorphism between the Z and W NORs detected here supports the notion that recombination is significantly reduced in this region to such an extent (perhaps even suppressed) that the NOR may have been subsumed into the non-recombining region from what was presumably the ancient pseudoautosomal region (Otto et al. 2011). Furthermore, the amplification of the rDNA sequences most likely occurred in association with the accumulation of the repetitive DNA sequences comprising the C-positive heterochromatin. Given that an identical pattern is seen in *A. spinifera* and *P. sinensis*, we propose that this reduction in recombination must have arisen at least ~95 mya (Fig. 1) at the divergence of these two genera, but the effect it may have on the function of the NOR still needs to be determined. Direct comparison of sex-linked orthologous genes in these two taxa will help test this hypothesis.

Importantly, the morphology of the ZZ/ZW sex chromosome system in *A. spinifera* appears to be orthologous with that previously described for *P. sinensis* (Kawai et al. 2007; Figs. 3c and 4g–i). This indicates that an

identical ZZ/ZW system is shared by *A. spinifera* and *P. sinensis* which arose at least ~95 mya and has since been retained unchanged. The lack of any noticeable divergence suggests that this ZZ/ZW may be ancestral to the entire family and, thus, could date to ~155 mya when Trionychidae split from Carettochelyidae. Additional sampling within Trionychidae to include other taxa besides *A. spinifera* and *P. sinensis* is needed to test this hypothesis. Our findings counter the idea that the differentiation of a pair of microchromosomes to sex chromosomes in *P. sinensis* was a spurious event representing an intermediate stage of sex chromosome differentiation as previously postulated by Kawai et al. (2007). The high degree of conservation of the trionychid sex chromosomes is surprising given that divergent XX/XY systems have evolved in Chelidae, between *E. macquarii*, *C. longicollis*, and *A. radiolata* (Sharma et al. 1975; Ezaz et al. 2006; Martinez et al. 2008) which are separated by similar or even less evolutionary time compared to *A. spinifera* and *P. sinensis* (Fig. 1). Other turtles such as *S. crassicolis* (Carr and Bickham 1981) and *P. smithii* (Sharma et al. 1975) that diverged even more recently (~40 mya; Valenzuela and Adams 2011) exhibit contrasting sex chromosome systems (XX/XY and ZZ/ZW, respectively), likely representing independent evolutionary events. Thus, insufficient time cannot account for the lack of divergence between *P. sinensis* and *A. spinifera*, and other forces must be responsible for this conservation. One possibility is the lack of genetic variation (or phylogenetic inertia) upon which natural selection or drift can act. Alternatively, stabilizing selection may be responsible for maintaining these identical sex chromosome systems and preventing their divergence. It has also been suggested that ZZ/ZW systems are less labile than XX/XY (Organ and Janes 2008). Finer scale sampling within and across turtle families is needed to unambiguously test these alternative hypotheses.

In summary, we identified a ZZ/ZW sex chromosome system in the GSD spiny softshell turtle *A. spinifera* that is identical to that identified in another softshell turtle, *P. sinensis*. Female *A. spinifera* (and *P. sinensis*) exhibits a NOR dimorphism which makes the female karyotype easily identifiable using metaphase chromosome analysis and can serve as a sex-specific diagnostic marker. The ability to reliably determine the genotypic sex prior to sexual maturity will be of great utility in future ecological, developmental, and evolutionary studies in softshell turtles. Our findings shed

light on the evolution of the remarkable diversity of sex-determining mechanisms in vertebrates. Further comparisons between trionychid softshell turtles (which have morphologically a conserved ZW system) and chelid side-neck turtles (which have highly divergent XY systems) may provide key insights into the causes and consequences of the strikingly different levels of sex chromosome diversification observed between these groups. Further research in these and other GSD turtles will help decipher the drivers of the evolution and structure of sex chromosomes including the identification of master sex-determining genes in turtles.

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