Discovery of Putative XX/XY Male Heterogamety in *Emydura subglobosa* Turtles Exposes a Novel Trajectory of Sex Chromosome Evolution in *Emydura*

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Keywords

Comparative genome hybridization · Cryptic heteromorphic sex chromosome evolution · Genotypic sex determination · Molecular cytogenetics · Pleurodiran chelid turtles · Y sex chromosome autosome fusion

Abstract

The discovery of sex chromosome systems in non-model organisms has elicited growing recognition that sex chromosomes evolved via diverse paths that are not fully elucidated. Lineages with labile sex determination, such as turtles, hold critical cues, yet data are skewed toward hide-neck turtles (suborder Cryptodira) and scant for side-neck turtles (suborder Pleurodira). Here, we used classic and molecular cytogenetics to investigate *Emydura subglobosa* (ESU), an unstudied side-neck turtle with genotypic sex determination from the family Chelidae, where extensive morphological divergence exists among XX/XY systems. Our data represent the first cytogenetic description for ESU. Similarities were found between ESU and *E. macquarii* (EMA), such as identical chromosome number ($2n = 50$), a single and dimorphic nucleolus organizer region (NOR) localized in a microchromosome pair (ESU14) of both sexes (detected via FISH of 18S rDNA). Only the larger NOR is active (detected by silver staining). As in

EMA, comparative genome hybridization revealed putative macro XX/XY chromosomes in ESU (the 4th largest pair). Our comparative analyses and revaluation of previous data strongly support the hypothesis that *Emydura*'s XX/XY system evolved via fusion of an ancestral micro-Y (retained by *Chelodina longicollis*) onto a macro-autosome. This evolutionary trajectory differs from the purported independent evolution of XX/XY from separate ancestral autosomes in *Chelodina* and *Emydura* that was previously reported. Our data permit dating this Y-autosome fusion to at least the split of *Emydura* around 45 Mya and add critical information about the evolution of the remarkable diversity of sex-determining mechanisms in turtles, reptiles, and vertebrates.

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Sex chromosomes are fundamental in many species for the development of males or females, including vertebrates, and are known to play an important role in the evolution of sex ratios, sexual selection, sexual dimorphism, and sexual conflict in species with genotypic sex determination (GSD) [Bachtrog et al., 2011]. Sex chromosomes are defined by the presence of a sex-determining region containing a sex-determining factor, and strong selection favors the reduction of recombination in

Table 1. GSD turtles with characterized sex chromosome systems

Turtle species	Technique	XX/XY	ZZ/ZW	Homo-morph	Hetero-morph	Macro-chromosome	Micro-chromosome	Reference
<i>Glyptemys insculpta</i>	CGH	+		+		+		Montiel et al. [2017]
<i>Glyptemys muhlenbergii</i>	PCR	+						Literman et al. [2017]
<i>Siebenrockiella crassicollis</i>	Giemsa	+		+		+		Carr and Bickham [1981]
<i>Pangshura smithii</i>	Giemsa		Over-turned		Over-turned	Over-turned		Sharma et al. [1975]; Mazzoleni et al. [2019]
<i>Staurotypus triporcatus</i>	Giemsa	+			+	+		Bull et al. [1974]; Sites et al. [1979]
<i>Staurotypus salvinii</i>	Giemsa	+			+	+		Bull et al. [1974]; Sites et al. [1979]
<i>Apalone spinifera</i>	CGH		+		+		+	Badenhorst et al. [2013]
<i>Pelodiscus sinensis</i>	CGH		+		+		+	Kawai et al. [2007]
<i>Acanthochelys radiolata</i>	Giemsa	+			+	+		McBee et al. [1985]
<i>Emydura macquarii</i>	CGH	+		+		+		Martinez et al. [2008]
<i>Chelodina longicollis</i>	CGH	+		+			+	Ezaz et al. [2006]

the heterogametic sex between the sex-determining region and nearby sexually antagonistic alleles that have differential fitness effects on males versus females [Bachtrog et al., 2011; Gschwend et al., 2012]. Current models of sex chromosome evolution propose that sex chromosomes originate from autosomes [Charlesworth and Charlesworth, 2000] and have done so multiple times independently in diverse lineages across the tree of life [Bachtrog et al., 2011, 2014]. Growing data from non-model organisms are changing our understanding of sex chromosome evolution by challenging theoretical paradigms derived mostly from comparative karyotypic research on organisms with well-differentiated sex chromosomes, such as insects, mammals, and birds [Bachtrog et al., 2011]. Thus, the discovery of additional sex chromosome systems is critical if we are to decipher the evolution of sex-determining mechanisms and associated traits [The Tree of Sex Consortium et al., 2014]. Sex chromosomes and sex determination in lower vertebrates such as reptiles remain poorly known. Yet, reptiles represent a group with highly variable modes of sex determination [Sarre et al., 2004; The Tree of Sex Consortium et al., 2014; Wang et al., 2015], ranging from chromosomal GSD as in other vertebrates, to temperature-dependent sex determination (TSD) in which the offspring's sex is determined by the environmental temperature during a window of embryonic development when the gonads form [Bull, 1980; Valenzuela and Lance, 2004]. Reptilian sex determination spans intermediate mechanisms [Valenzuela et al., 2003; Sarre et al., 2004] for which some bona

fide examples exist [Shine et al., 2002; Radder et al., 2008; Holleley et al., 2015], while others were refuted empirically [Valenzuela et al., 2014; Mu et al., 2015].

Of the about 360 turtle species recognized today [Turtle Taxonomy Working Group et al., 2017], the sex-determining mechanism has been studied in only about 90 (~25%) [The Tree of Sex Consortium et al., 2014]. Most species examined to date exhibit TSD with no sex chromosomes [Valenzuela et al., 2014], and the sex chromosome system of male (XX/XY) or female heterogamety (ZZ/ZW) has been characterized in only 10 of the 19 reported GSD turtles (Table 1) [Literman et al., 2017; Montiel et al., 2017 and references therein], because the case of ZZ/ZW was recently overturned in *Pangshura smithii* as the original report was due to a karyotyping error [Mazzoleni et al., 2019]. The scarcity of sex chromosome information in turtles is due in part to the fact that some sex chromosomes are poorly differentiated in shape and size and thus are undetectable by classical cytogenetics. These cryptic sex chromosomes are only recognizable visually by the use of modern molecular cytogenetic techniques, such as comparative genome hybridization (CGH) [Ezaz et al., 2006]. CGH has facilitated the characterization of sex chromosomes across a wide range of reptiles by detecting subtle genomic differences between males and females [Ezaz et al., 2005, 2006, 2009; Matsubara et al., 2013], even in relatively young sex chromosome systems [Montiel et al., 2017]. In turtles, CGH permitted discovering micro sex chromosome in 3 turtle species [*Chelodina longicollis* (Ezaz et al., 2006), *Pelodiscus*

sinensis (Kawai et al., 2007), and *Apalone spinifera* (Badenhorst et al., 2013)] as well as macro sex chromosomes in 2 others [*E. macquarii* (Martinez et al., 2008) and *Glyptemys insculpta* (Montiel et al., 2017)]. Other methods, such as cross-species PCR amplification of sex-linked molecular markers, uncovered the heterogametic system in the highly endangered and congeneric *G. muhlenbergii* [Litterman et al., 2017].

In turtles, sex determination co-evolves with diploid number, such that transitions in sex determination are associated with an acceleration in the evolution of the chromosome number [Valenzuela and Adams, 2011]. Comparative studies reconstruct TSD as the ancestral condition in turtles from which GSD and sex chromosomes evolved independently at least 5 times [Pokorná and Kratochvíl, 2009; Valenzuela and Adams, 2011; Sabath et al., 2016], with 2 likely reversals back to TSD [Valenzuela and Adams, 2011; Litterman et al., 2018]. However, these working hypotheses may change as additional sex chromosome systems are discovered.

Also unexplained is the evolution of the morphological diversity of XY chromosomes in chelid turtles. A previous study documented a remarkable case of morphological stasis in the sex chromosomes of softshell turtles (Trionychidae) whose heterogametic ZW chromosomes have changed relatively little over ~95 My of evolution between *P. sinensis* and *A. spinifera* [Badenhorst et al., 2013]. In contrast, XY chromosomes in chelid turtles have diverged substantially in their shape and size during a similar time frame (~90 My) that separates the lineages whose sex chromosomes have been characterized cytogenetically. Indeed, all 9 chelid turtles studied to date lack TSD [Bull and Legler, 1980], and XX/XY systems were reported in 3 of them, including macro XY chromosomes with a smaller Y in *Acanthochelys radiolata* [McBee et al., 1985], homomorphic micro XY chromosomes with divergent Y content in *C. longicollis* [Ezaz et al., 2006], and macro XY chromosomes where the male-specific region is restricted to the tip of the larger Y in *E. macquarii* [Martinez et al., 2008].

To help alleviate the scarcity of data on sex chromosomes in side-neck turtles, we used CGH to characterize the system of *E. subglobosa* by identification of sex-specific regions that revealed its type of heterogamety. *E. subglobosa*, the New Guinea red-bellied short-necked turtle, is commonly found in Australia, Indonesia, and Papua New Guinea [Georges et al., 1993; Turtle Taxonomy Working Group et al., 2017]. The species was formally described as *Euchelymys subglobosa* [Kreff, 1876] and later assigned to the genus *Emydura* [Boulenger,

1888]. *E. subglobosa* diverged from *E. macquarii* about 30 Mya [Minh et al., 2013]. As all other studied chelids, *E. subglobosa* has GSD [Ewert et al., 2004], but no cytogenetic study has been conducted for this taxon and its sex chromosome system is unknown. We then combined this new information and published data on other relatives [Ezaz et al., 2006; Martinez et al., 2008; Matsubara et al., 2015] in a phylogenetic framework to shed light on the evolution of the sex chromosomes in this lineage of chelid turtles.

Materials and Methods

Animal and Tissue Sampling

Ten *E. subglobosa* (ESU) hatchlings were obtained from a pet store. The sex of each animal was determined by gonadal inspection under a dissecting scope. There were 9 females and 1 male among the 10 hatchlings. Muscle tissue biopsies were collected from the tail tip or limbs of each animal and used for primary cell culture.

Cell Culture and Chromosome Preparation

Cell culture and molecular cytogenetic protocols followed standard procedures for the detection of cryptic sex chromosomes in turtles [Martinez et al., 2008; Badenhorst et al., 2013]. Primary fibroblast cell cultures were established from tail clip tissue of the 1 male and 9 females, digested with collagenase (Gibco), and cultured using a medium composed of 1:1 RPMI 1640:Leibowitz media (Gibco) supplemented with 15% fetal bovine serum (One Shot, Gibco), 2 mM L-glutamine (Gibco), and 1% antibiotic-antimycotic solution (Gibco). Cell cultures were incubated at 30°C without CO₂ supplementation. Four hours prior to harvesting, 10 µg/mL colcemid (KaryoMAX®, Gibco) was added to the cultures. Metaphase chromosomes were harvested after hypotonic exposure and fixed in 3:1 methanol:acetic acid. Cell suspensions were dropped onto glass slides, fixed with formaldehyde, and air dried.

Comparative Genome Hybridization

Male and female genomic DNA (gDNA) from 1 male and 8 female *E. subglobosa* was separately extracted and labeled by standard nick-translation (Abbott Molecular) using either biotin-16-dUTP or digoxigenin-11-dUTP (Roche) following the manufacturer's instructions. CGH was performed as described elsewhere [Badenhorst et al., 2013; Montiel et al., 2017]. Briefly, chromosome slides were incubated at 65°C for 2 h, denatured 1 min 45 s at 70°C in 70% formamide/2× SSC, dehydrated through an ethanol series, and air dried. For each slide, a 15-µL mixture containing 250–500 ng of digoxigenin-11-dUTP female and biotin-16-dUTP male was co-precipitated with 5–10 µg boiled gDNA from male or female (as competitor) and 20 µL glycogen (as carrier). This mixture was hybridized to each slide in a humid chamber at 37°C for 3 days. Slides were washed post-hybridization twice, first in 0.4× SSC/0.3% Tween-20 for 2 min at 60°C, and then in 2× SSC/0.1% Tween-20 for 1 min at room temperature. A 200-µL solution of 4XT/relevant-antibody (fluorescein-conjugated avidin or anti-digoxigenin-rhodamine) was used for fluoro-

Fig. 1. G-banded metaphase chromosomes and CGH of *E. subglobosa*. G-banded metaphase and karyotype of male (**A**, **C**) and female (**B**, **D**) individuals show autosomes and sex chromosomes (4th largest chromosomes pair) ordered by size. CGH on male (**E**) and female (**F**) *E. subglobosa* chromosome spreads. Female DNA was detected with anti-digoxigenin-rhodamine (red) and male DNA with FITC avidin (green). Scale bars, 10 μ m.

chrome detection at 37°C for 1 h. Slides were subsequently washed 3 times in 4XT (4 $\times$ SSC/0.05% Tween^{TM}-20) at 37°C and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were then mounted using an antifade solution (Vectashield). Although it was likely that females were the homogametic sex given the existence of a XX/XY system in the congeneric species *E. macquarii* [Martinez et al., 2008], reciprocal experiments were conducted using the gDNA of both sexes as competitor, and each sex-by-color combination was tested to rule out all possible systems (XY, ZW, lack of sex chromosomes) as described in Valenzuela et al. [2014].

G-Banding, (ATCC)_n Microsatellite, and NOR Mapping

Chromosomes were analyzed by conventional staining via G-banding following the protocols described in Badenhorst et al. [2013]. G-banding was carried out using the standard enzymatic trypsin/Giemsa technique [Seabright, 1971].

A DNA microsatellite repeat probe with a (ATCC)_n motif was generated for FISH by non-template PCR amplification using

primers (ATCC)₄ and (GGAT)₄ as described for other repeat motifs [Ijdo et al., 1991] with slight modifications. Briefly, PCR reactions were performed in 50 μ L volumes containing 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 200 μ M of each dNTP, 0.1 μ M of each primer, and 2 units of *Taq* polymerase. Amplification consisted of first 10 cycles, each 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by 30 cycles, each 1 min at 94°C, 30 s at 60°C, and 90 s at 72°C, and 1 final step of 5 min at 72°C. This (ATCC)_n DNA probe was labeled with biotin-16-dUTP, and FISH was conducted as described in Montiel et al. [2016].

The visualization of NORs (the genomic regions containing the genes for the 5.8S, 18S, and 28S ribosomal subunits) was investigated in 2 ways, (1) by silver staining (Ag-NOR) which detects the active NORs on mitotic chromosomes [Howell and Black, 1980], and (2) by FISH of ribosomal genes using a turtle-specific 18S DNA fragment labeled with biotin-16-dUTP as described earlier [Badenhorst et al., 2013].

Image Analysis

Images were taken with a Leica DFC365 FX camera attached to an Olympus BX41 fluorescence microscope and analyzed using CytoVision® cytogenetic analysis system. A minimum of 10 complete metaphase spreads were analyzed for each specimen per experiment. Chromosomes were distinguished by morphology, size, and DAPI-banding.

Results

Karyotype of *E. subglobosa*

Our cytogenetic analyses by G-banding revealed an identical overall karyotype in all metaphases of all 10 *E. subglobosa* individuals studied. The chromosome number was $2n = 50$ in all cases (Fig. 1), including 13 pairs of bi-armed macrochromosomes (8 metacentric, 3 sub-metacentric, and 2 subtelocentric chromosome pairs), and 12 pairs of microchromosomes (which are morphologically indistinguishable and were assigned to pairs as best as possible). As a difference between male and female karyotypes, the fourth largest chromosome pair in males (ESU4) showed a discernible but somewhat subtle heteromorphism, with a sub-telocentric chromosome (putative X chromosome) and a slightly larger sub-metacentric chromosome (putative Y chromosome). The 2 homologs of the fourth largest pair in all 9 females were always subtelocentric and homomorphic, suggesting the presence of an XX/XY sex chromosome system in *E. subglobosa*.

Comparative Genome Hybridization

A male-specific region was identified in the male on the tip of the p arm of one homolog of the ESU4 pair, the putative Y chromosome, where only a single color (fluorophore) was detected corresponding to the male genomic DNA. Instead, the remainder of the ESUY, the ESUX, and all other chromosomes in the male painted uniformly with both male and female DNA. All females lacked this male-specific (Y-specific) hybridization pattern, and instead, the male and female DNA hybridized equally on all female chromosomes, consistent with a XX-female pattern (Fig. 1E, F). These CGH results support the existence of a XX/XY male heterogametic system in this species.

(ATCC)_n Microsatellite FISH Mapping

Hybridization using the DNA probe containing the (ATCC)_n microsatellite motif revealed a block on the telomeric region of a single chromosome of the male ESU4 where this repeat sequence has expanded (Fig. 2A). This ATCC-rich region co-localizes with the sex-specific region detected by CGH in the male metaphases. The

(ATCC)_n probe produced no hybridization signal in any other chromosomes of the male nor in any female.

NOR Mapping by Silver Staining and 18S rDNA FISH

A single NOR was detected in a pair of relatively larger micro-autosomes (ESU14) by both 18S rDNA FISH (Fig. 2B, C) and by silver staining (Ag-NOR) (Fig. 2D, E). The FISH signals revealed a dimorphism in the size of the 18S rDNA clusters within individuals, such that one chromosome of the pair exhibited a larger FISH signal and the other homolog a smaller cluster (Fig. 2B, C). This was true in both males and females (Fig. 2B, C) such that no sexual dimorphism was detected in the NOR. Notably, the Ag-NOR staining was detected exclusively in the chromosome harboring the larger 18S cluster, indicating that only the larger NOR is active whereas the smaller NOR is silent.

Discussion

E. subglobosa and *E. macquarii* Share a Virtually Identical Karyotype and Heterogamety

In this study, we examined for the first time the karyotype of *E. subglobosa*, a pleurodiran turtle from Australia in the family Chelidae, and tested alternative hypotheses about the causes and timing of the evolution of the sex chromosome system of the genus *Emydura*. Our results indicate that *E. subglobosa* has a diploid chromosome number of $2n = 50$, the most common chromosome number among turtles. This is consistent with other data reported for all Australian chelid genera [Killebrew, 1976; Bull and Legler, 1980]. Our data help ameliorate the gap in cytological studies of pleurodiran (side-necked) turtles, a suborder comprised of Pelomedusidae, Podocnemididae, and Chelidae. We found that the G-banded karyotype of *E. subglobosa* is similar to that of *E. macquarii* in general chromosome morphology, with only slight or no differences, as reported previously within and among closely-related pleurodiran genera [Bull and Legler, 1980].

GSD species, including turtles, may have homomorphic sex chromosomes that are identical in morphology but with genetically differentiated content as seen in the *C. longicollis* XY system [Ezaz et al., 2006]. In contrast, the closely related *E. macquarii* exhibits a slightly heteromorphic XY system [Martinez et al., 2008] (Fig. 3). Here, we detected a XX/XY system in *E. subglobosa*, similar to the sex chromosomes of *E. macquarii*, with the male-specific signal located on the tip of the slightly larger Y chromo-

Fig. 2. Localization of Y-specific (ATCC)_n microsatellite motif and NOR in *E. subglobosa*. **A** (ATCC)_n repeat probe labeled with biotin-dUTP (green). 18S rDNA FISH in male **(B)** and female **(C)** using probes labeled with biotin-dUTP (green). Silver staining detected the active NOR in male **(D)** and female **(E)**. Scale bars, 10 μm.

some. This restricted topology of the male-specific region may indicate an early stage of sex chromosome differentiation, perhaps because not enough time has elapsed for the small sex-limited region to further expand or for the Y to degenerate. However, this similarity in size and shape of the XY system shared by *E. macquarii* and *E. subglobosa* indicates that this sex chromosome system is at least about 45 My old, having originated at the split of *Emydura* from other chelids at the latest. We note that among the 10 individuals examined here, only 1 was male, and molecular cytogenetic analyses typically include more

than a single individual to identify the sex chromosome system with confidence in a species. However, given that the sex chromosomes of *E. macquarii* are well characterized [Martinez et al., 2008; Matsubara et al., 2015] and that the male *E. subglobosa* examined here exhibited an identical XY system to *E. macquarii* (after using DNA from 8 different females to test for potential sex reversals) [Valenzuela et al., 2014] lends credence to our conclusions. However, our results should be taken as tentative until more males are examined in future studies. Importantly, our study is not unique in using a single individu-

Fig. 3. Alternative hypotheses of sex chromosome evolution in *Emydura* chelid turtles, and phylogenetic relationships among turtles. **A** Under the first hypothesis, a micro-Y chromosome (as observed in *C. longicollis*) present in the common ancestor of *Emydura* and *Chelodina* turtles fused onto the end of a macro-autosome, forming a neo-XY system in *Emydura*'s lineage (as observed in *E. macquarii*), while the ancestral micro-X was lost (alternatively, both micro X and Y may have fused with each autosomal homolog). **B** Under the second hypothesis, a macro XY (as observed in *Emydura*) present in the common ancestor of *Emydura* and *Chelodina* underwent fission at the tips of the large X and Y, generating the micro XY observed in *C. longicollis*. **C** Under the third hypothesis, the XY systems of *E. macquarii* and *C. longicollis*

originated from distinct autosomes present in their common ancestor and thus are not homologous. Panels **D**, **E**, and **F** illustrate the hybridization results expected under hypotheses A, B, and C (respectively), as detailed in the discussion. **G** Phylogenetic relationships among chelid turtles with known sex determination along with their chromosome number. Reported sex chromosomes of *Acanthochelys radiolata*, *E. macquarii*, and *C. longicollis* are shown on the right, along with 2 proposed hypotheses tested here for *E. subglobosa* which would be supported by finding a XX/XY system in *E. subglobosa* identical to *E. macquarii* (hyp-1) or to *C. longicollis* (hyp-2). Thunder symbols denote putative timing of sex chromosome evolution under hyp-1 (yellow) or hyp-2 (pink).

al of one or both sexes to identify the sex chromosome system. A single male and female were utilized to describe the sex chromosome system in some *Anolis* lizards [Rovatsos et al., 2014a] and in over 67 other squamates [Rovatsos et al., 2014b, 2015].

Our FISH mapping revealed the accumulation of a (ATCC)_n microsatellite repeats on the male-limited region of the Y chromosome in *E. subglobosa*. The expansion of repetitive sequences, such as microsatellite repeats, on differentiated highly heterochromatic sex chromosomes (Y and W) as reported in many reptiles and birds, is strongly associated with the process of differentiation and heterochromatinization that often characterize sex chromosomes, and may differ among species [Pokorna et al., 2011; Matsubara et al., 2015]. This particular (ATCC)_n repeat motif is also found in the heterochromatic regions of the Y of *E. macquarii* and *C. longicollis* turtles, the Y of the pink-tailed worm-lizard, and the W of tiger snakes [Matsubara et al., 2015].

Finally, we identified a single NOR in *E. subglobosa* by 18S rDNA FISH, located in a micro-autosome pair (ESU14), which is dimorphic in size within individuals but equally so in both sexes (the 18S rDNA cluster is larger in one homolog than the other). This condition is also observed in the closely related *E. macquarii* [Montiel et al., 2016]. The NOR in *Emydura* is autosomal as occurs in several other turtles, located on macro-autosomes (in *Chelydra serpentina*, *Carettochelys insculpta*, *Pelomedusa subrufa*, and *Podocnemis unifilis*) and on micro-autosomes (in *Chrysemys picta*, *Trachemys scripta*, *G. insculpta*, *Sternotherus odoratus*, *C. oblonga*, and *E. macquarii*). In contrast, NORs are sex-linked in other turtles (ZW of *A. spinifera* and *P. sinensis*, and X of *Staurotypus triporcatus*) [Montiel et al., 2016]. Dimorphic NORs exist in *P. sinensis* where the W carries a much larger 18S rDNA gene cluster compared to the Z [Kawai et al., 2007; Badenhorst et al., 2013].

Revisiting the Evolution of Sex Chromosomes in *Emydura* and *Chelodina*

To better understand the evolution of the XX/XY system in *E. subglobosa*, we next revisited previously proposed alternative hypotheses about the evolution of sex chromosomes in *Emydura* [Martinez et al., 2008] (Fig. 3A–C) in light of our findings and of current chromosome painting data from other studies [Matsubara et al., 2015]. First, we noted that the wide variation in chromosome number across turtles points to drastic changes in genome architecture and rearrangements in this order, which are linked to transitions in the mechanism of sex determination [Valenzuela and Adams, 2011; Montiel et al., 2016]. Phylogenetic reconstruction analysis estimated the diploid chromosome number for chelids as $2n = 52$, from which $2n = 50$ evolved in *Emydura* and $2n = 54$ evolved in *Chelodina* [Montiel et al., 2016] (Fig. 3), likely by a chromosomal fusion and fission, respectively. Whether any of these particular fusion and fission events are directly involved in sex chromosome evolution remains unknown, yet they could alter the relative position and regulation of genes governing sexual development as occurs by chromosomal rearrangements observed in some turtles [Lee et al., 2019].

Based on data of the diploid number and sex chromosome morphology from *E. macquarii* and *C. longicollis*, several testable hypotheses were proposed about the evolution of sex chromosomes in *Emydura* and *Chelodina* [Martinez et al., 2008] (Fig. 3). Under the first hypothesis, *C. longicollis* micro XY may represent the ancestral sex chromosome system for *Emydura* and *Chelodina*, which perhaps fused with an autosomal pair at the split of *Emydura*'s lineage (Fig. 3A). This sex chromosome to autosome fusion would have generated a neoX/neoY system, giving rise to the macro XY seen in *E. macquarii* [Martinez et al., 2008] and *E. subglobosa* (this study) and reducing the diploid number from $2n = 52$ to $2n = 50$ (Fig. 3A, G). Perhaps only a Y-autosome fusion occurred (followed by the eventual loss of the micro X) as occurs often [Pennell et al., 2015], or more unlikely, perhaps both X and Y fused to an autosomal pair.

Under the second hypothesis [Martinez et al., 2008], the macro XY of *E. macquarii* and *E. subglobosa* may represent the ancestral state for *Emydura* and *Chelodina* from which the micro system observed in *C. longicollis* evolved via the fission of the tips of the macro XY where the male-specific signal is located, generating the micro XY in *C. longicollis* (Fig. 3B, G). These 2 scenarios imply that the sex chromosomes of *Emydura* and *C.*

longicollis (or at least their Y-specific regions) are homologous.

The third hypothesis proposed that the XY systems of *Emydura* and *Chelodina* evolved independently from distinct autosomes [Martinez et al., 2008] (Fig. 3C).

A recent chromosome painting study reported support for the third hypothesis [Matsubara et al., 2015], but our re-evaluation of their data leads to the conclusion that the trajectory of sex chromosome evolution in *Emydura* actually followed the first hypothesis. In particular, under the first hypothesis (Fig. 3A), it is predicted that a probe generated from *E. macquarii* Y chromosome (EMAY) would paint both EMAX and EMAY in their entirety, whereas a probe from EMAX would paint the entire EMAX and most EMAY except for the male-limited Y tip (Fig. 3D). Importantly, a 2-color EMAX+EMAY cocktail would paint the homologous autosomal pair of macrochromosomes in *C. longicollis* (CLOA) plus the homologous micro CLOY, but not CLOX if the ancestral micro-X was lost in *Emydura*'s lineage (Fig. 3D). Notably, the chromosome painting data from Matsubara et al. [2015] concord exactly with these predictions (Fig. 3D), thus supporting the scenario of the first hypothesis (Fig. 3A). Further, our data suggest that this Y-autosome fusion occurred at least 45 Mya in *Emydura*, or perhaps earlier (since $2n = 50$ is shared by *Emydura*'s close relatives; Fig. 3G). In contrast, the conclusion that the results from Matsubara et al. [2015] are supportive of the notion that 2 ancestral autosome pairs were co-opted independently as XY in *Emydura* and *Chelodina* separately (Fig. 3C) is mistaken. This error becomes evident because, under that third scenario, the EMAY probe is not expected to paint the analogous CLOY (Fig. 3F), yet that was the result of the experiments of Matsubara et al. [2015] (Figure 4E in Matsubara et al. [2015]). The other alternative hypothesis (Fig. 3B) that the ancestor of *Emydura* and *Chelodina* possessed a macro XY (as observed in *Emydura*) and that the tips of these large X and Y broke up generating the micro XY observed in *C. longicollis* is also refuted by the chromosome painting data from Matsubara et al. [2015], because their results differ from the prediction that under this scenario the EMAX and EMAY probes should both paint CLOX (Fig. 3E).

In conclusion, the present study alleviates the scarcity of cytogenetic information from side-neck turtles by providing the first characterization of the karyotype of *E. subglobosa*. Our data permitted the identification of an XX/XY system in this GSD chelid turtle that is identi-

cal to that found in its congener *E. macquarii* [Martinez et al., 2008]. Revisiting previous data from chromosomal painting revealed a new evolutionary trajectory than previously proposed [Matsubara et al., 2015], supporting the notion that *Emydura*'s sex chromosome system evolved via the fusion of an ancestral micro-Y chromosome (as present in *C. longicollis*) onto a macro-autosome, and our data permitted timing this fusion to at least the split of *Emydura* around 45 Mya (Fig. 3G). Further data are needed on other GSD close relatives such as *Elseya* and *Elusor* to test if the Y-autosome fusion dates back as far as 70 Mya or 90 Mya when these lineages separated (Fig. 3G). It is also unknown whether the distinct XY found in *Acantochelys radiolata* represents a unique sex chromosome system or one shared by other GSD close relatives such as *Mesoclemmys* and *Phrynos*. Our findings of the discovery of the sex chromosome system of *E. subglobosa* add critical information about the evolution of the remarkable diversity of sex-determining mechanisms in turtles.

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Acknowledgements

Special thanks go to B.A. Mizoguchi for her assistance with hatchling care and gonadal inspection. We thank J.M. Serb, T.A. Peterson, T.A. Heath, and M. Hufford at Iowa State University for their comments. This work was funded in part by NSF grant IOS-1555999 to N.V.

Statement of Ethics

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University.

Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

N.V. and L.S.L. conceived the study, analyzed data, interpreted results, and wrote the manuscript. E.M.M. conducted the cell culture. L.S.L. collected the data.

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