

An XX/XY heteromorphic sex chromosome system in the Australian chelid turtle *Emydura macquarii*: A new piece in the puzzle of sex chromosome evolution in turtles

Pedro Alonzo Martinez^{1*}, Tariq Ezaz², Nicole Valenzuela¹, Arthur Georges³ & Jennifer A. Marshall Graves²
¹Department of Ecology, Evolution, and Organismal Biology, Iowa State University, 253 Bessey Hall, Ames, IA 50011, USA; Tel: +1-515-294-1285; Fax: +1-515-294-1337; E-mail: pete1025@iastate.edu; ²Comparative Genomics Group, Research School of Biological Sciences, The Australian National University, Canberra, ACT 2601, Australia; ³Institute for Applied Ecology, University of Canberra, Canberra, ACT 2601, Australia
*Correspondence

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Abstract

Chromosomal sex determination is the prevalent system found in animals but is rare among turtles. In fact, heteromorphic sex chromosomes are known in only seven of the turtles possessing genotypic sex determination (GSD), two of which correspond to cryptic sex microchromosomes detectable only with high-resolution cytogenetic techniques. Sex chromosomes were undetected in previous studies of *Emydura macquarii*, a GSD side-necked turtle. Using comparative genomic hybridization (CGH) and GTG-banding, a heteromorphic XX/XY sex chromosome system was detected in *E. macquarii*. The Y chromosome appears submetacentric and somewhat larger than the metacentric X, the first such report for turtles. CGH revealed a male-specific chromosomal region, which appeared heteromorphic using GTG-banding, and was restricted to the telomeric region of the p arm. Based on our observations and the current phylogeny of chelid turtles, we hypothesize that the sex chromosomes of *E. macquarii* might be the result of a translocation of an ancestral Y microchromosome as found in a turtle belonging to a sister clade, *Chelodina longicollis*, onto the tip of an autosome. However, in the absence of data from an outgroup, the opposite (fission of a large XY into an autosome and a micro-XY) is theoretically equally likely. Alternatively, the sex chromosome systems of *E. macquarii* and *C. longicollis* may have evolved independently. We discuss the potential causes and consequences of such putative chromosome rearrangements in the evolution of sex chromosomes and sex-determining systems of turtles in general.

Abbreviations

BrdU	5'-bromo-2'-deoxyuridine
CGH	comparative genomic hybridization
CCD	charge-coupled device
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
ESD	environmental sex determination
FBS	fetal bovine serum
FISH	fluorescence in-situ hybridization
GSD	genotypic sex determination
GTG	Giemsa trypsin banding

G-banding	Giemsa banding
gDNA	genomic deoxyribonucleic acid
PHA	phytohaemagglutinin
SSC	sodium chloride sodium citrate

Introduction

Sexually reproducing organisms employ an astonishing diversity of mechanisms to produce males and

females, ranging from systems under strict genetic control (genotypic sex determination, GSD) to systems under strict environmental control (environmental sex determination, ESD) (Bull 1983, Solari 1994, Valenzuela *et al.* 2003, Valenzuela & Lance 2004, Ezaz *et al.* 2006). GSD mechanisms include highly dimorphic or undifferentiated sex chromosome systems in male (XX/XY) or female (ZZ/ZW) heterogamety, which is the most common mechanism among animals, but more complex systems are also known (Bull 1983). While XX/XY is found in all mammals with some notable exceptions (e.g. Grützner *et al.* 2004, Just *et al.* 2007), and ZZ/ZW is found in all birds, both systems coexist among amphibians, fish and reptiles, and co-occur with ESD in fish and reptiles (Bull 1983, Solari 1994, Valenzuela & Lance 2004). This variety of sex-determining mechanisms found among reptiles makes them an ideal group in which to study sex chromosome evolution.

Theoretical models propose that sex chromosomes originate when a sex-determining mutation arises in a pair of autosomes (Ohno 1967, Charlesworth 1991). This initial step is followed by the accumulation near the sex-determining locus of additional mutations conferring some sex-specific advantage, and by decreased recombination, sometimes involving chromosomal inversions or rearrangements (Bull 1983, Ohno 1967, Modi & Crews 2005). This process can lead to the formation of two morphologically distinct sex chromosomes, exhibiting different patterns of heterochromatin accumulation and deletions, and to the degeneration of the non-recombining heterogametic sex chromosome (Y or W) owing to its higher mutation accumulation rate and ineffective selection (Ohno 1967, Vallender & Lahn 2004, Waters *et al.* 2007). The degeneration of the heterogametic sex chromosome may ultimately cause its extinction (Graves 2002, 2006), and a new pair of sex chromosomes may be formed *de novo* by the reiteration of the same process (Charlesworth *et al.* 2005, Just *et al.* 2007), or by the translocation of an ancestral sex-determining gene onto an autosome (e.g. van Doorn & Kirkpatrick 2007). Examples of nascent sex chromosomes are known both in animals (e.g. Almeida-Toledo *et al.* 2000, Peichel *et al.* 2004, Just *et al.* 2007) and in plants (e.g. Liu *et al.* 2004, Ming *et al.* 2007). Degeneration of the heterogametic sex chromosome is not ubiquitous, however (e.g. Sites *et al.* 1979), and some species are known in which the Y chromosome is larger than the X (e.g.

some *Drosophila*, polychaetes, frogs and plants; Lewis & John 1963, Sato & Ikeda 1992, Solari 1994, Matsunaga & Kawano 2001).

The rates of recombination and the evolutionary dynamics of genes housed in sex chromosomes and autosomes differ such that processes like Muller's ratchet, genetic hitchhiking and intralocus conflict apply differently to them (Charlesworth *et al.* 1987). Consequently, the evolution of sex chromosomes has significant consequences for the evolution of sex ratio, sexual dimorphism, sexual selection, sexual conflict, and ultimately, speciation and extinction (e.g. Berry & Shine 1980, Rice 1984, Lindholm & Breden 2002, Edwards *et al.* 2005, Saether *et al.* 2007). Therefore, it is critical to correctly identify sex chromosome systems if we are to understand their evolution and the evolution of these related traits.

In turtles, only seven species have been reported to have heteromorphic sex chromosomes. These include five XX/XY systems in *Acanthochelys radiolata* (McBee *et al.* 1985), *Staurotypus salvinii* and *S. triporcatus* (Bull *et al.* 1974), *Siebenrockiella crassicollis* (Carr & Bickham 1981), and *Chelodina longicollis* (Ezaz *et al.* 2006), and two ZZ/ZW systems in *Pangshura (Kachuga) smithii* (Sharma *et al.* 1975), and *Pelodiscus sinensis* (Kawai *et al.* 2007). In most of these species, sex chromosomes are macro sex chromosomes, with the exceptions of *C. longicollis* (Ezaz *et al.* 2006) and more recently, *P. sinensis* (Kawai *et al.* 2007). The first report of sex microchromosomes in the agamid lizard, *Pogona vitticeps* (ZZ/ZW: Ezaz *et al.* 2005), raised the question of the prevalence of microchromosomes in GSD reptiles as cryptic sex chromosomes that are only detectable with high-resolution cytogenetic techniques such as comparative genome hybridization (CGH). Here we address this question by using CGH to identify sex chromosomes of a chelid turtle, *Emydura macquarii*, a species closely related to *C. longicollis* (Georges & Thomson 2006) with GSD but no visible sex chromosomes.

E. macquarii is a side-necked turtle endemic to the eastern coast of Australia (Georges & Thomson 2006). Incubation experiments showed that *E. macquarii* has GSD (Thompson 1988), yet no heteromorphic sex chromosomes were detected in previous cytogenetic studies (Killebrew 1976, Bull & Legler 1980). Because of the close relationship between the *E. macquarii* and *C. longicollis* clades (Figure 1, Georges & Thomson 2006), characterizing the sex

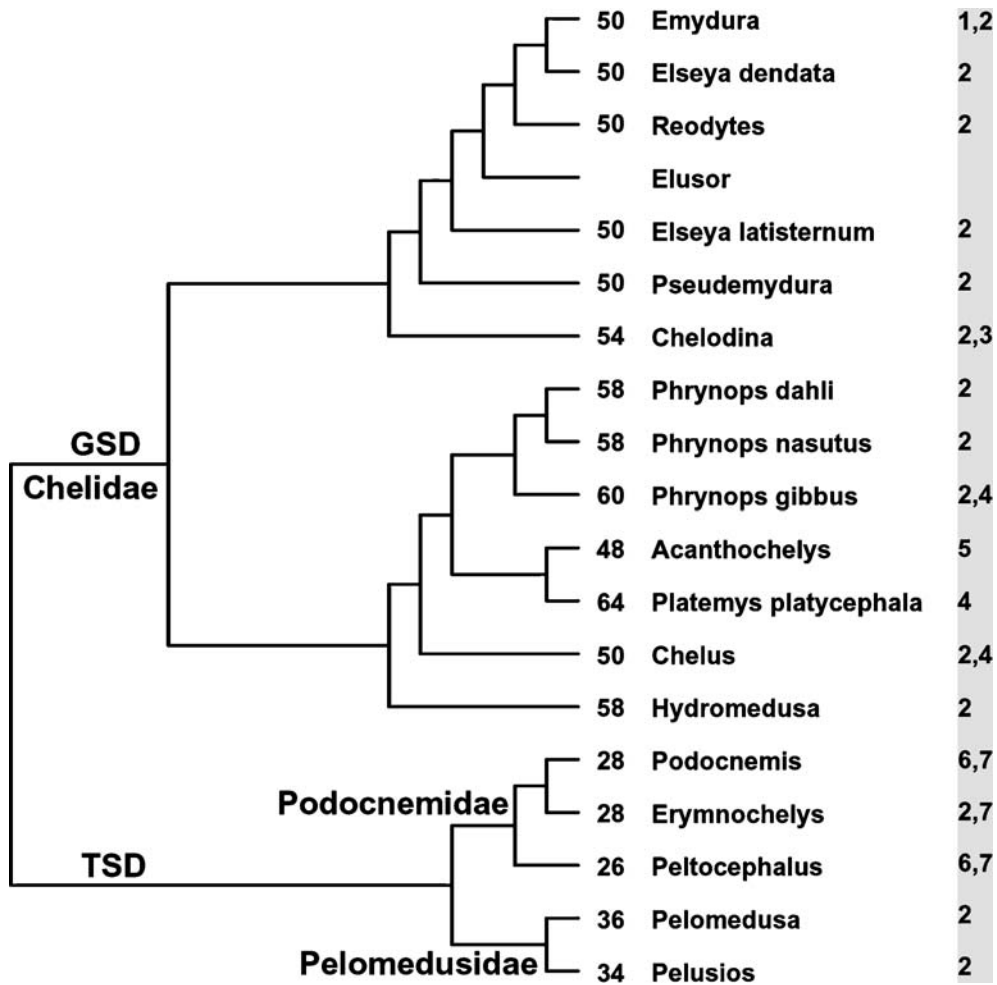


Figure 1. Current phylogenetic hypothesis (pruned tree) of the relationships of Pleurodiran turtles, based on Georges & Thomson (2006) and Iverson *et al.* (2007). Chromosome numbers are indicated in the left column and sources in the shaded column. 1=Killebrew (1976); 2=Bull and Legler (1980), 3=Ezaz *et al.* (2006), 4=Barros *et al.* (1976), 5=Rhodin *et al.* (1984), 6=Ayres *et al.* (1969), 7=Rhodin *et al.* (1978).

chromosome system in *E. macquarii* is an important step that will help assessing the prevalence of micro sex chromosome systems and reconstructing the evolutionary history of sex-determining mechanisms in chelid turtles, and ultimately in reptiles.

Materials and methods

Animals

Six adult male and six adult female *E. macquarii* were captured at Wentworth, New South Wales, Australia, at the junction of the Darling and Murray Rivers (34° 6' 27" S, 141° 55' 5" E) by D. Bower and

K. Hodges of the Institute for Applied Ecology, University of Canberra, Australia, under the following permits for the transport, housing and bleeding: Department of Environment and Heritage – Permit to Undertake Scientific Research Q25104, Ministerial Fisheries Exemption No. 9901935, University of Canberra Ethics Clearance CEAE 07–08. Blood samples were provided by D. Bower. The sex of each animal was determined by examination of sexually dimorphic external morphology.

Blood culture and chromosome preparation

Metaphase spreads of mitotic chromosomes were obtained from 5–6-day cultures of peripheral blood

leukocytes as described in Ezaz *et al.* (2005) with a few modifications as follows. Briefly, 0.5 ml of blood was collected from each individual from the jugular vein with a 25-gauge needle attached to a 1 ml disposable syringe. No heparin was used when removing the blood. Aliquots of 100 μ l blood were placed into tubes with 10 μ l of 10 000 U/ml sodium heparin (Sigma, St. Louis, MO, USA) and later into 2 ml of Dulbecco's Modified Eagle Medium (DMEM, GIBCO-Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. The medium was supplemented with 100 U/ml penicillin (Multicell, Wisent. Inc., Quebec, Canada), 100 μ g/ml streptomycin (Multicell) and 4% phytohaemagglutinin M (PHA M; Sigma). Cultures were incubated at 28°C for 5–6 days with 5% CO₂. Eight and six hours before harvesting, 35 μ g/ml 5'-bromo-2'-deoxyuridine (BrdU; Sigma) and 75 ng/ml colcemid (Roche, Indianapolis, IN, USA) were added to each culture. Metaphases were fixed in 3:1 methanol–acetic acid. Cell suspension was dropped onto wet cold slides pre-chilled at 4°C, and slides were dried on a hotplate at 60°C. For DAPI (4',6-diamidino-2-phenylindole) staining, slides were immersed into a DAPI solution with a concentration of 0.001 mg/ml for 30 s, and then mounted with Vectashield (Vector Laboratories Burlingame, CA, USA).

DNA extraction and labelling

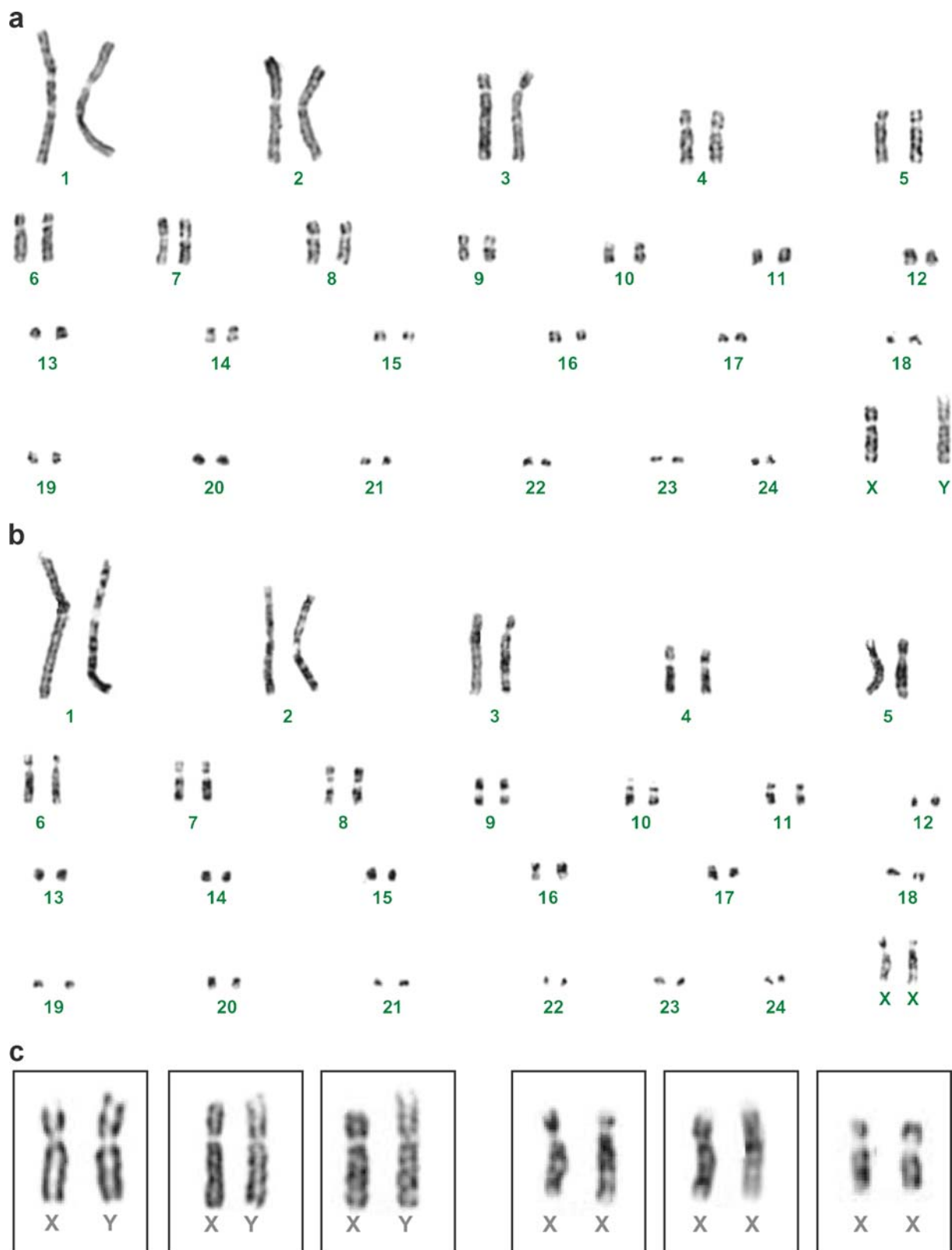
Total genomic DNA (gDNA) was extracted from whole blood following Ezaz *et al.* (2004). Nick translation was used to label total gDNA. Male total gDNA was labelled with SpectrumRed-dUTP (Vysis, Abbott, Des Plaines, IL, USA), while female total gDNA was labelled with SpectrumGreen-dUTP (Vysis, Inc.). The reverse was also done such that each sex was tested with both colours.

Comparative genomic hybridization (CGH) and chromosome banding

The comparative genomic hybridization (CGH) followed Ezaz *et al.* (2005) with a few modifications.

Metaphase chromosome slides were aged at –80°C overnight and were denatured for 1.5 min at 70°C in 70% formamide and 2 \times SSC, dehydrated through a 70%, 80% and 100% ethanol series for 3 min each, and air-dried at room temperature until hybridization. For each slide that was made, 250–500 ng of *E. macquarii* SpectrumRed-labelled male and SpectrumGreen-labelled female DNA were co-precipitated with 5 μ g of boiled *E. macquarii* female DNA, along with 20 μ g of glycogen (as carrier) and 3 volumes of 100% ethanol. Reciprocal experiments were done using male DNA competitor since the homogametic sex was not known at the onset of the experiment. Following an overnight co-precipitation at –20°C, the probe mix was centrifuged at 15 700 g at 4°C. The supernatant was discarded and the probe DNA pellet was resuspended in 40 μ l of 37°C pre-warmed hybridization buffer (50% formamide, 10% dextran sulfate, 2 \times SSC, 40 mmol/l sodium phosphate pH 7.0 and 1 \times Denhardt's solution) and resuspended for at least 30 min at 37°C. The hybridization mixture was denatured at 70°C for 8 min, immediately placed on ice for 2 min, and 16 μ l of the probe mixture was placed as a single drop per slide. Slides were covered with cover glass (22 mm \times 22 mm), sealed with rubber cement and placed inside a humid hybridization chamber at 37°C for 3 days. Slides were washed at 55°C in 0.4 \times SSC, 0.3% Tween 20 for 2 min, followed by a second wash in 2 \times SSC, 0.1% Tween 20 for 1 min at room temperature. Slides were left to air dry at room temperature, counter-stained with DAPI and then mounted with Vectashield (Vector Laboratories) as described earlier. Images were captured with a Zeiss Axioplan epifluorescence microscope with a CCD camera (RT-spot, Diagnostic Instrument of Sterling Heights, Michigan, USA) using filters 02, 10, or 15 from the Zeiss fluorescence filter set. The camera was controlled by an Apple Macintosh Computer. IPLab scientific imaging software (V.3.9, Scanalytics, BD Biosciences, Rockville, MD, USA) was used to capture greyscale images, and to superimpose and merge the source CGH images into one colour image.

Figure 2. G-banded metaphase karyotypes of *E. macquarii*: (a) male, (b) female. 2n=50 (24 macrochromosomes and 26 microchromosomes). The male karyotype (a) shows the autosomes, as well as the X and Y pair, with a heteromorphism on the p arm of the Y. The female karyotype (b) shows perfect matching of all macrochromosome homologues and the absence of any heteromorphic pair, as is expected for the XX system. The heteromorphic p arm of the Y chromosomes compared with the X is also evident in the sex chromosome pairs from 3 male and 3 female karyotypes shown on panel (c).



GTG- banding

Freshly dropped slides were aged for 2 days at 60°C. For G-banding, slides were treated in 0.5% trypsin-EDTA (Gibco) for 2 min and placed into 10% FBS (Gibco) as a rinse for 5 s to stop the enzymatic reaction. Slides were then placed into a 5% Giemsa solution (Gurr's buffer, pH 6.8) for 3 min. Subsequently, they were rinsed with distilled water and dried with an air blower. Slides were examined under a Zeiss microscope. Images were captured with a Cytovision software system version 3.9 from Applied Imaging, on a PC computer with Windows XP.

Results

Here we describe the karyotype of *E. macquarii*, the discovery of a heteromorphic pair, and confirmation by CGH that this pair differs between sexes.

Emydura macquarii karyotype

GTG-banded mitotic karyotypes of three male and three female *E. macquarii* were examined (Figure 2). A total of 10 mitotic metaphase spreads were analysed for each sex. The karyotypes obtained from males (Figure 2a) and females (Figure 2b) confirmed the diploid number of $2n=50$ previously reported for *E. macquarii* (Killebrew 1976, Bull & Legler 1980). The transition between macrochromosomes and microchromosomes was not sharp, such that at least 12 pairs of macrochromosomes were evident in all metaphase spreads (including the sex chromosome pair), consistent with Bull & Legler (1980) (e.g. Figure 2b), while 13 pairs of macrochromosomes and the 12 pairs of microchromosomes were apparent in some spreads, consistent with Killebrew (1976) (e.g. Figure 2a). The macrochromosomes include 7 pairs of metacentric and 5 pairs of submetacentric chromosomes (Figure 2a, b). The other 13 pairs were all microchromosomes (Figure 2a, b). GTG-banding patterns were used to pair every macrochromosome with its homologue (Figure 2), but did not allow pairing of all microchromosomes because of their size.

Comparison of the karyotypes from males and females revealed a morphological difference in a pair

of macrochromosomes between the sexes. All male metaphases analysed showed a heteromorphic pair in males that differed in size and centromere position that was homomorphic in females, suggesting an XX female : XY male system. In males, the male-specific chromosome (the Y) was slightly larger than its partner (the X) and had a longer short arm, so that it was nearly metacentric (Figures 2a, 2c and 3a). In females, both homologues of the presumptive X were virtually identical in length (average ratio of chromosome length=1.01, range 1–1.05). In males the Y was 1.2 times the size of the X in its total length on average (range 1.1–1.26). The distal region of Yp was heterochromatic in most males, staining noticeably lighter than on its homologue through G-banding. This difference was never seen in females. This identifies a male-specific Y-chromosome, revealing the presence of a male heterogametic sex chromosome system (XX female : XY male) in *E. macquarii*.

Mitotic metaphase spreads from two males and two females were DAPI-stained and 10 spreads were analysed for each sex. In DAPI-stained karyotypes (Figure 3), only the four largest macrochromosomes can be distinguished clearly in both males and females, as the absence of banding precludes pairing of the other macrochromosomes. The fourth largest pair corresponds to the sex chromosome pair. The heteromorphic region detected with GTG-banding at the tip of the Y chromosome appeared DAPI faint (Figure 3a).

Comparative genomic hybridization (CGH) of *Emydura macquarii*

CGH was performed on cells from two males and one female *E. macquarii*, using differentially labelled and co-precipitated male and female total gDNA probe to perform fluorescent in-situ hybridization (FISH). A sex-specific pattern was observed (Figure 4). Males exhibited a differential hybridization signal on the fourth largest macrochromosome, the same macrochromosome identified as the Y chromosome by GTG-banding (Figure 4c,d). The upper portion of the short arm of the Y chromosome exhibited a bright hybridization signal that was absent from its homologue, the X. The differential signal at the tip of the Yp coincides with the heteromorphic region detected using GTG-banding. This pattern was observed in all the male cells examined, but none on the metaphases from females (Figure 4g,h).

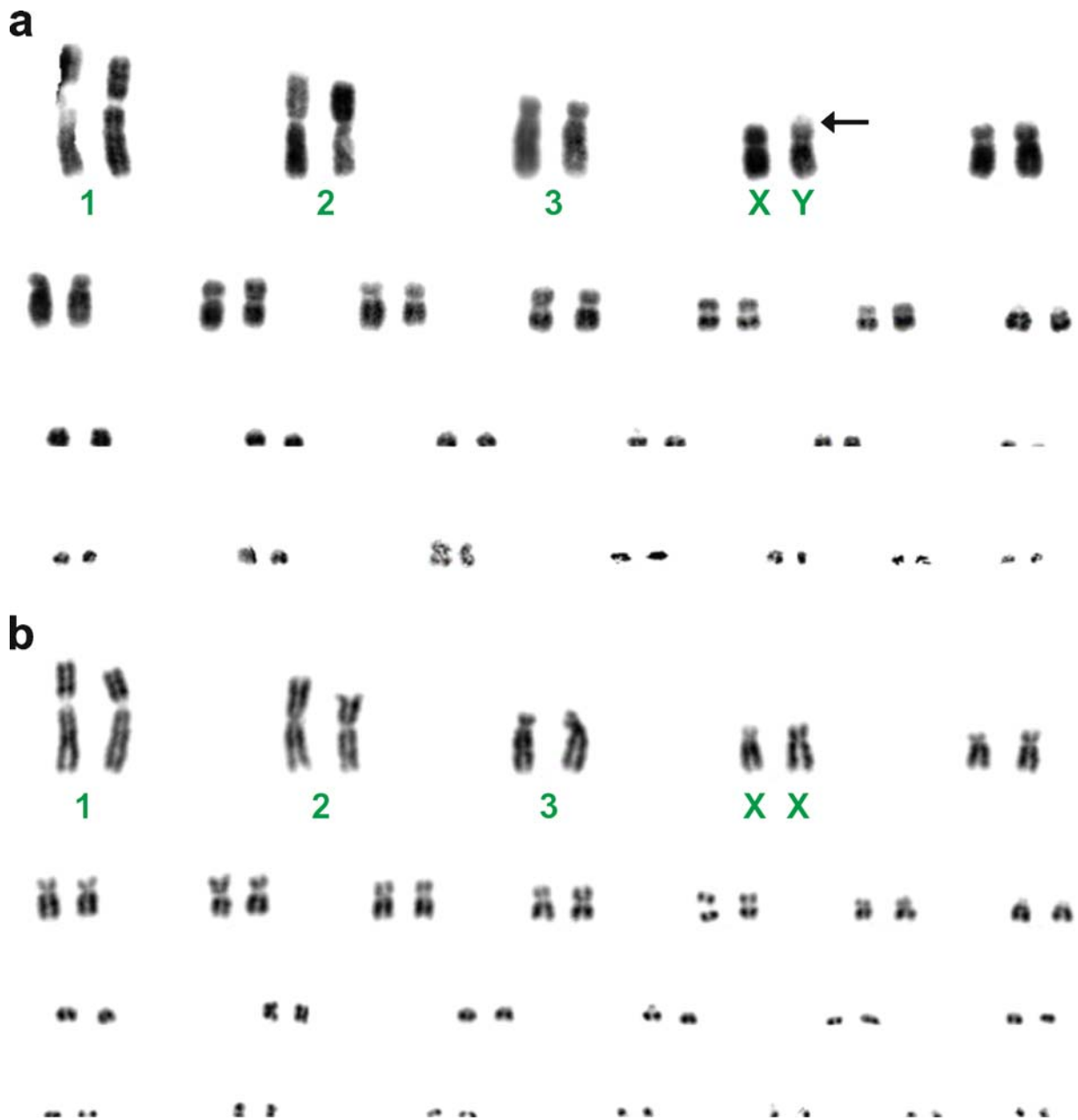


Figure 3. DAPI-stained metaphase karyotypes of *E. macquarii*: (a) male, (b) female. Note the DAPI-faint tip of the p-arm of the Y chromosome in the male.

The localization of the male-specific CGH and GTG-banding signals in the same chromosomal region confirms that this is the Y chromosome of *E. macquarii*, and that this species possesses a male heterogametic sex chromosome system (XX/XY). The CGH differential signal was consistent in all metaphases examined.

Discussion

Sex chromosome systems are highly labile and elucidation of the processes responsible for generating this diversity is essential to understanding their evolution. Reptile sex-determining systems are particularly variable, and include environmental and

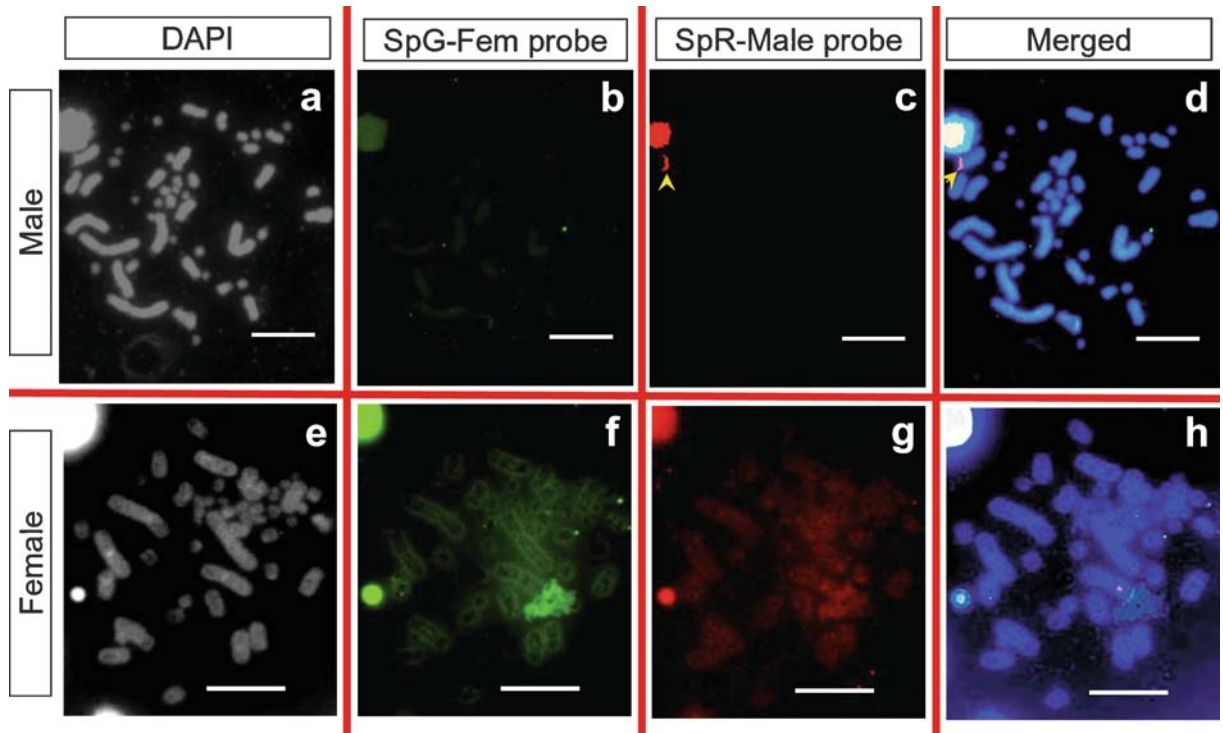


Figure 4. CGH in the chromosomes of *Emydura macquarii*: male (upper row) and female (bottom row). (a, e) DAPI-stained metaphase chromosome spread; (b, f) SpectrumGreen-labelled female total genomic DNA; (c, g) SpectrumRed-labelled male total genomic DNA; (d, h) merged images. Arrow indicates the Y chromosome. Scale bar=10 μ m.

genetic sex determination, only some of which are accompanied by cytologically detectable sex chromosome differentiation. The application of high-resolution cytogenetic techniques is helping reveal an increasing number of otherwise cryptic sex chromosome systems in GSD reptiles whose sex chromosomes show no visible gross morphological differentiation (Ezaz *et al.* 2005, 2006, Kawai *et al.* 2007).

Here we examined the karyotype of *E. macquarii*, a turtle known to have GSD from incubation experiments (Thompson 1988) but reported as lacking heteromorphic sex chromosomes as determined from previous cytogenetic studies (Killebrew 1976, Bull & Legler 1980). Using CGH and GTG-banding we were able to confirm *E. macquarii*'s diploid number ($2n=50$) (Killebrew 1976, Bull & Legler 1980), as well as that it possesses heteromorphic sex chromosomes.

In contrast to previous studies, our GTG-banding and CGH results permitted the detection of a heteromorphic region in a pair of macrochromosomes of *E. macquarii* that was not evident in the orcein-

stained and non-banded chromosomes of a single unsexed animal published by Killebrew (1976) or the karyotypes stained solely with Giemsa, also lacking any banding (Bull & Legler 1980) for *Emydura*.

Of ~20 turtle species known to possess GSD (Valenzuela 2004), only seven have been identified to have differentiated sex chromosomes. Five of these are easily detectable by conventional cytogenetic techniques (Bull *et al.* 1974, Sharma *et al.* 1975, Carr & Bickham 1981, McBee *et al.* 1985), but two species have cryptic sex microchromosomes that were revealed only by special cytogenetic techniques (Ezaz *et al.* 2006, Kawai *et al.* 2007).

Surprisingly, the sex chromosomes in *E. macquarii* comprise a pair of macrochromosomes, unlike its close relative *C. longicollis*, which possesses cryptic sex microchromosomes (Ezaz *et al.* 2006). It is interesting to consider the relationship of the macro sex chromosomes of *E. macquarii* to the microchromosomes of *C. longicollis*, a closely related turtle from which it diverged <50 Mya (Near *et al.* 2005). An ancestral Y microchromosome may have

been fused or translocated onto the end of an autosome, rendering it a compound neo-Y chromosome. Since the number of chromosomes is the same in males and females, the X must have either been lost (very unlikely) or fused to the same autosome, which would occur if a translocation occurred within a pseudoautosomal pairing region at a stage of partial differentiation of the ancestral micro XY. However, in the absence of data from an outgroup, the opposite (fission of a large XY into an autosome and a micro-XY) is theoretically equally likely. Alternatively, the sex chromosome systems of *E. macquarii* and *C. longicollis* may have evolved independently starting from different sex-determining mutations on different autosome pairs. This might be less likely, since the species diverged recently, yet the almost complete differentiation of X and Y, at least in *C. longicollis* (Ezaz *et al.* 2006), is typical of an ancient sex pair. These alternative hypotheses could be tested by assessing the homology of *E. macquarii* and *C. longicollis* using DNA from an isolated *C. longicollis* Y (and X) to paint onto an *E. macquarii* metaphase and analysing where hybridization occurs, as well as by comparative banding analyses.

The Y chromosome of *Emydura* is larger than the X (Figure 2), an observation made in other disparate animal and plant taxa (Lewis & John 1963, Sato & Ikeda 1992, Solari 1994, Matsunaga & Kawano 2001), but not previously in turtles. This suggests the addition to and/or amplification of repetitive sequences on the male-specific Y chromosome, which is consistent with the homology of the Y with the bulk of the X, as well as the pale staining of the male-specific distal region of the Y. Other species in which the Y chromosome is also larger than the X are *Drosophila melanogaster* (Lewis & John 1963), *Neanthes japonica* polychaete worms (Sato & Ikeda 1992), *Gastrotheca riobambae* frogs (Solari 1994) and *Silene latifolia* plants (Matsunaga & Kawano 2001). In anuran frogs, *Crinia bilingua* and *Discoglossus pictus*, the W is also larger than the Z (White 1973, Solari 1994). The same was reported originally for *Xenopus laevis*, but later studies have found sex chromosomes to be homomorphic in this species and other congeners (White 1973, Schmid & Steinlein 1991, and references therein). Increases in the size of the Y chromosome or expansion of specific regions compared to the X have been ascribed in some plants and fish to duplications, or the insertion of transposable elements or autosomal sequences (Ming *et al.* 2007).

Other important questions about turtle sex chromosomes emerge that warrant further research. For instance, a rearrangement of the Y and/or X chromosome (whether fusion, translocation or amplification) could have profound changes in the *cis*- and *trans*-regulation of the genes contained in these chromosomes (e.g. Carvalho & Clark 2005). Furthermore, if any of these genes (such as the sex-determining factor) are ecologically important, a rearrangement of this kind could be responsible for the evolutionary split of the *Emydura* lineage. Chromosome speciation has been documented in a number of taxa (e.g. Masly *et al.* 2006). The process that is responsible for the spread and fixation of large chromosomal mutations, such as chromosomal translocations that arise in a single individual and typically reduces the reproductive fitness of its bearer has stimulated much theoretical debate (Coghlan *et al.* 2005). While several models describe how chromosome speciation may occur (Ayala & Coluzzi 2005, Coghlan *et al.* 2005), others propose that chromosomal rearrangements may be the result rather than the cause of speciation (White 1968, Coghlan *et al.* 2005).

In summary, we identified an XX/XY sex chromosome system in a GSD chelid turtle involving a macrochromosome pair. The male-specific Y chromosome is larger than the X, and contains a male-specific heterochromatic region on the p arm that was detected by GTG banding and comparative genome hybridization. The larger Y is probably due to the addition and amplification of repetitive sequences. These large sex chromosomes may be related to the sex microchromosomes of the closely related *C. longicollis* (Georges & Thomson 2006), either by fusion of an ancestral XY microchromosome pair to an autosome or by the reverse: fission of an XY microchromosome from a larger ancestral sex macrochromosome pair. Alternatively, the two XY chromosome systems may have evolved independently from unrelated macro- and microchromosomes. Further research is needed to test these alternative hypotheses and to demonstrate the homology between *E. macquarii*'s male-specific region and *C. longicollis* Y microchromosome. Finer taxonomic sampling using high-resolution cytogenetic techniques such as CGH, accurate banding methods, and gene mapping will be essential to reveal a more accurate picture of the prevalence and evolution of vertebrate sex-determining mechanisms in a phylogenetic context.

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