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An XX/XY sex microchromosome system in a freshwater turtle, *Chelodina longicollis* (Testudines: Chelidae) with genetic sex determination

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Abstract

Heteromorphic sex chromosomes are rare in turtles, having been described in only four species. Like many turtle species, the Australian freshwater turtle *Chelodina longicollis* has genetic sex determination, but no distinguishable (heteromorphic) sex chromosomes were identified in a previous karyotyping study. We used comparative genomic hybridization (CGH) to show that *C. longicollis* has an XX/XY system of chromosomal sex determination, involving a pair of microchromosomes. C-banding and reverse fluorescent staining also distinguished microchromosomes with different banding patterns in males and females in ~70% cells examined. GTG-banding did not reveal any heteromorphic chromosomes, and no replication asynchrony on the X or Y microchromosomes was observed using replication banding. We conclude that there is a very small sequence difference between X and Y chromosomes in this species, a difference that is consistently detectable only by high-resolution molecular cytogenetic techniques, such as CGH. This is the first time a pair of microchromosomes has been identified as the sex chromosomes in a turtle species.

Introduction

Sex determination in vertebrates may be triggered by a variety of genetic and environmental mechanisms. The most-studied taxa, the birds and mammals, have genetic sex determination (GSD) with different but conserved sex chromosomal systems (ZW female:ZZ male in birds and XX female:XY male in mammals). Other groups such as reptiles show greater diversity, encompassing GSD with XY or ZW sex chromosomal systems (with and without heteromorphic sex chromosomes) and temperature-dependent sex determination (TSD; reviewed in Valenzuela & Lance 2004, Sarre *et al*. 2004).

The identification of the heterogametic sex is difficult in GSD species with no distinguishable sex chromosomes, as found in many species of fish, amphibians, and some snakes and lizards, and nearly

all turtle species. Of the 155 turtle species karyotyped (61% of the 254 known turtle species), sex chromosomes have been described in only four species. The sex chromosomes identified were all macrochromosomes, and included both XX/XY and ZW/ZZ sex chromosomal systems (Olmo & Signorino 2005; Table 1). A single chelid turtle *Acanthochelys radiolata* was reported to have heteromorphic sex chromosomes (with a XX/XY sex chromosomal system) but since only one male was examined, study involving individuals from both sexes is required to confirm the male heterogamety in this species (McBee *et al*. 1985).

The differentiation of sex chromosomes seems to follow a general rule that the heterogametic member of the pair degenerates (Charlesworth 1996). One interesting feature of turtle sex chromosomes is that, unlike other vertebrates, the Y chromosome is not always the most differentiated sex chromosome (Olmo 1986). For example, in *Staurotypus* the X has undergone differentiation from the progenitor pair, while the Y does not show any apparent modification (Sites *et al*. 1979).

Incubation of eggs at a range of constant and natural fluctuating temperature regimes has demonstrated that *Chelodina longicollis*, an Australian freshwater turtle, has genetic sex determination (Georges 1988, Palmer-Allen *et al*. 1991). However, the only previous karyotyping study of this species found no heteromorphic sex chromosomes (Bull and Legler 1978). Thus this species was considered to belong with the very many other GSD reptiles with homomorphic sex chromosomes.

However, the application of modern cytogenetic techniques such as comparative genomic hybridization (CGH) may reveal cryptic sex chromosomes in such reptiles. For example, recent work on the dragon lizard *Pogona vitticeps*, another GSD reptile (Viets *et al*. 1994, Harlow 2001) in which the initial karyotyping study identified no heteromorphic sex chromosomes (Witten 1983), has differentiated the cryptic sex chromosomes in this species (Ezaz *et al*. 2005). The sex chromosomes of *P. vitticeps* are cryptic in the sense that they eluded discovery by traditional light microscopic examination and banding, only to be discovered by the more sensitive molecular cytogenetic technique of CGH, which revealed female heterogamety in this species (ZZ/ZW sex microchromosomes). Once discovered, closer examination by traditional banding approaches could distinguish the sex chromosome pair (Ezaz *et al*. 2005). The technique of CGH has also successfully been adapted to demonstrate the sex chromosomal differences in a diverse group of animals with varying degree of sex chromosomal differences (Traut *et al*. 1999, 2001, Barzotti *et al*. 2000).

Table 1. A summary of sex chromosomes in turtle families (modified from Olmo & Signorino 2005 and Peter Uetz 2005).

Families	No. of genus/ no. of species	Species with sex chromosomes/species karyotyped	Species	Types of sex chromosomal systems
Cheloniidae	5/7	0/6	—	—
Dermochelyidae	1/1	0/1	—	—
Chelydridae	3/3	0/3		
Emydidae	11/70	2/66	*Kachuga smithii*	ZW/ZZ
			Siebenrockiella crassicollis	XX/XY
Testudinidae	13/~50	0/17	—	—
Dermatemydidae	1/1	0/1	—	—
Kinosternidae	4/~23	2/17	*Staurotypus salvinii*	XX/XY
			S. triporcatus	XX/XY
Carettochelyidae	1/1	0/1	—	—
Trionychidae	14/25	0/8	—	—
Chelidae	11/~40	0/23	*Chelodina longicollis**	XX/XY
			*Acanthochelys radiolata***	
Pelomedusidae	5/26	0/5	—	—
Podocnemididae	3/8	0/8	—	—
Totals	72/254	4/155		

* present study; ** based on the observation of only one male (McBee *et al*. 1985).

Here we report the application of the molecular cytogenetic technique of comparative genomic hybridization (CGH) and several banding techniques (C-banding, GTG-banding, late replication banding and reverse fluorescence staining) to differentiate the cryptic sex chromosomes of *C. longicollis*.

Materials and methods

Animals

Five adult female and six adult male *Chelodina longicollis* were collected from two locations in New South Wales, Australia. Their phenotypic sex was determined by external morphology of the plastra as well as by examination of the gonads using laparoscopy.

Animal collection, handling, sampling and all other relevant procedures were performed following the guidelines of the Australian Capital Territory Animal Welfare Act 1992 (Section 40), and the permits and the licences issued by Environment ACT and the New South Wales State government (animal welfare permit no. S10661) and with the approval of the Australian National University Animal Experimentation Ethics Committee (Proposals R.CG.02.00 and R.CG.08.03) and the University of Canberra Animal Experimentation Ethics Committee (Proposal CEAE 04/04).

Blood culture and chromosome preparation

Mitotic metaphase chromosome spreads were prepared from short-term culture of whole blood as well as from peripheral blood leukocytes as described in Ezaz *et al*. (2005). Briefly, blood was collected from the external jugular vein with a heparinized (Heparin–sodium salt; Sigma) 25-gauge needle attached to a 1–2 ml disposable syringe. Mitotic and meiotic chromosomes were prepared as follows. Approximately 100 μl of heparinized blood was used to set up 2 ml culture in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (JRH Biosciences), 1 mg/ml L-Glutamine (Sigma), 10 μg/ml gentamycin (Multicell), 100 units/ml penicillin (Multicell), 100 μg/ml streptomycin (Multicell) and 3% phytohaemagglutinin M (PHA M; Sigma). Cultures were incubated at 30°C for 96–120 h in 5% CO_2 incubators. Six and four hours prior to harvesting, 35 μg/ml 5′-bromo-2′-deoxyuridine (BrdU; Sigma) and 75 ng/ml colcemid (Roche) were added to the culture respectively. Metaphase chromosomes were harvested and fixed in 3:1 methanol : acetic acid following the standard protocol (Verma and Babu 1995). Cell suspension was dropped onto glass slides and air-dried. For DAPI (4′,6-diamidino-2-phenylindole) staining, slides were mounted with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI.

Testes from two males were used for meiotic chromosome preparation following the protocol described in Ezaz *et al*. (2005). Briefly, the testicular tunica was removed in calcium- and magnesium-free phosphate buffered saline and the seminiferous tubules cut into small pieces using a sterile scalpel blade. These tissues were incubated in 75 mmol/L KCl for 30–45 min at 37°C or overnight at room temperature, and then fixed in 3 : 1 methanol : acetic acid. Cell suspension was prepared by dissolving a piece of tissue in equal volumes of freshly prepared 3 : 1 methanol : acetic acid and distilled water. The slides were prepared and DAPI stained as described earlier.

DNA extraction and labelling

Total genomic DNA was extracted from whole blood following the protocol of Ezaz *et al*. (2004). Nick translation was used to label total genomic DNA. The female total genomic DNA was labelled with SpectrumGreen-dUTP (Vysis, Inc.), while the male total genomic DNA was labelled with SpectrumRed-dUTP (Vysis, Inc.).

Comparative genomic hybridization (CGH) and chromosome banding

We followed the procedure of comparative genomic hybridization as described by Ezaz *et al*. (2005). Briefly, slides were denatured for 2–2.5 min at 70°C in 70% formamide, 2X SSC, dehydrated through an ethanol series, air-dried and kept at 37°C until probe hybridization. For each slide (made using one drop of cell solution), 250–500 ng of SpectrumGreen-labelled female and SpectrumRed-labelled male DNA was co-precipitated with (or without) 5–10 μg of boiled genomic DNA from the homogametic sex (as competitor), and 20 μg glycogen (as carrier).

Since the homogametic sex was not known, reciprocal experiments were performed using alternatively male and female DNA as competitor.

The co-precipitated probe DNA was resuspended in 20 µl hybridization buffer (50% formamide, 10% dextran sulphate, 2X SSC, 40 mmol/L sodium phosphate pH 7.0 and 1X Denhardt's solution). The hybridization mixture was denatured at 70°C for 10 minutes, rapidly chilled on ice for 2 min and then 18 µl of probe mixture was placed on a single drop on a slide and hybridized at 37°C in a humid chamber for 3 d. Slides were washed once at 60 ± 1°C in 0.4X SSC, 0.3% Tween 20 for 2 min followed by another wash at room temperature in 2X SSC, 0.1% Tween 20. Slides were then air dried and mounted with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 µg/ml DAPI. Images were captured using a Zeiss Axioplan epifluorescence microscope equipped with a CCD (charge-coupled device) camera (RT-Spot, Jackson instrument) using either filters 02, 10 and 15 from the Zeiss fluorescence filter set or the Pinkel filter set (Chroma technologies, filter set 8300). The camera was controlled by an Apple Macintosh computer. IPLab scientific imaging software (V.3.9, Scanalytics, Inc.) was used to capture grey scale images and to superimpose and to co-localize the source images into a colour image.

Chromosome banding and staining

GTG-banding, C-banding, replication banding and reverse fluorescent staining were performed as described by Ezaz *et al.* (2005). Briefly, freshly dropped or up to 10 d old slides aged at 37°C were used for GTG-banding. Slides were treated with 0.05% trypsin (Gibco BRL) solution (in 1X Dulbecco's PBS-CMF) for 15–60 s, then rinsed briefly in cold PBS-CMF (2–5°C, kept in refrigerator) and stained in 5% Giemsa (in Gurr's buffer, pH 6.8) for 5–8 min at room temperature, rinsed in distilled water, air-dried and then mounted with D.P.X. (Ajax Chemicals) neutral mounting medium.

For C-banding, slides were aged at room temperature for 2–3 d, soaked in 0.2N HCl for 40 min at room temperature, then treated with $Ba(OH)_2$ (Sigma) for 7 min at 50°C and finally 1 h at 60°C in 2X SSC. Slides were rinsed in distilled water and stained with 4% Giemsa in 0.1 mol/L phosphate buffer for 10–30 min at room temperature. After staining slides were rinsed in distilled water, air-dried, and mounted with D.P.X. (Ajax Chemicals) neutral mounting medium. For late replication banding, BrdU incorporated chromosome preparations were dropped onto microscope slides and were incubated overnight at 55°C. In the next morning, slides were immersed in methanol for several seconds and incubated for 3–5 min at 40°C in tetrasodium EDTA-Giemsa solution (3% Giemsa solution in 2% tetrasodium EDTA).

Reverse fluorescent chromosome staining was performed as described by Schweizer (1976). Briefly, 200–300 µl of 0.5 mg/ml chromomycin A3 (CA3) solution (in McIlvaine's buffer, pH 7.0) was placed on the slide and covered with a cover slip. Slides were incubated at room temperature in the dark in a humid chamber for 1–3 h, then rinsed in distilled water, air dried, and mounted with anti-fade medium Vectashield containing 1.5 µg/ml DAPI (Vector Laboratories). The slides were examined under a fluorescent microscope.

Results

Karyotypes of Chelodina longicollis

The DAPI-stained mitotic karyotypes of three males and two females were examined (Figure 1). A total of 40 mitotic metaphase chromosome spreads were counted for each individual. The chromosomes are arranged into three groups on the basis of the centromeres and sizes (Bickham 1975). Our study confirmed that the diploid chromosome complement of *C. longicollis* is 2n = 54, as described earlier by Bull and Legler (1980). There were 12 pairs of macrochromosomes and 15 pairs of microchromosomes, with a gradual decrease in sizes between macro and microchromosomes. The 12 macrochromosome pairs comprise 6 metacentric, 4 submetacentric and 2 acrocentric pairs. All the microchromosomes were DAPI faint except two pairs, which have very strong DAPI bands. The centromeres of the microchromosomes could not be detected accurately because of their size. Comparison of the karyotypes from males and females did not reveal the presence of any morphologically differentiated sex chromosomes (Figure 1a, b).

The meiotic chromosomes from the testes of three male *C. longicollis* were prepared and DAPI banding patterns from 25 cells were analysed. The first meiotic division/diakinesis from testis of *C. long-*

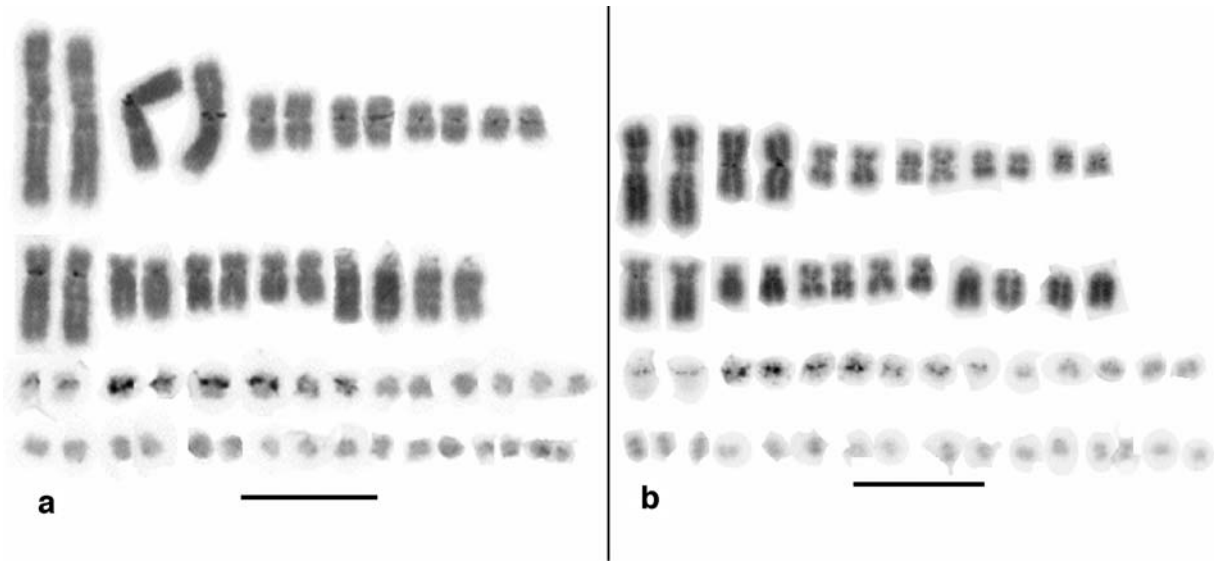

Figure 1. DAPI-stained metaphase karyotypes of *Chelodina longicollis* {2n = 54 (24 macrochromosomes + 30 microchromosomes)}. **a**: male metaphase karyotype; **b**: female metaphase karyotype. Scale bar indicates 10 μm.

icollis showed 27 pairs of chromosomes. No size or DAPI banding differences indicating heteromorphisms were observed in any of the pairs, nor were unpaired regions evident (Figure 2). Our very initial investigation involving C-banding differences between X and Y chromosomes in male meiosis failed to detect any banding difference between X and Y chromosomes in the very contracted microchromosomes (data not shown).

Comparative genomic hybridization (CGH)

CGH was performed for three male and two female *C. longicollis* and 40 cells were analysed for each

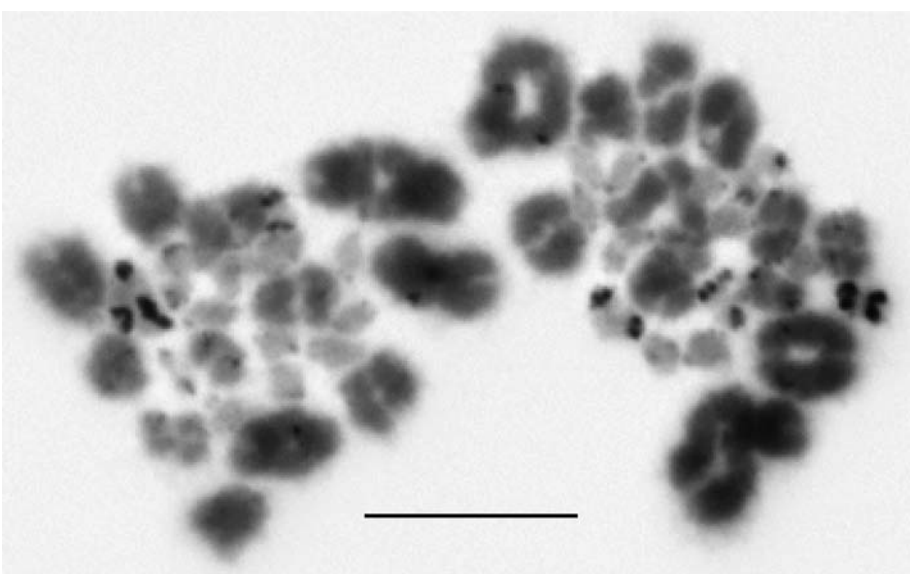

Figure 2. DAPI-stained meiotic prophase/diakinesis in male *Chelodina longicollis*, showing pairing between the homologous chromosomes in two cells. Scale bar indicates 10 μm.

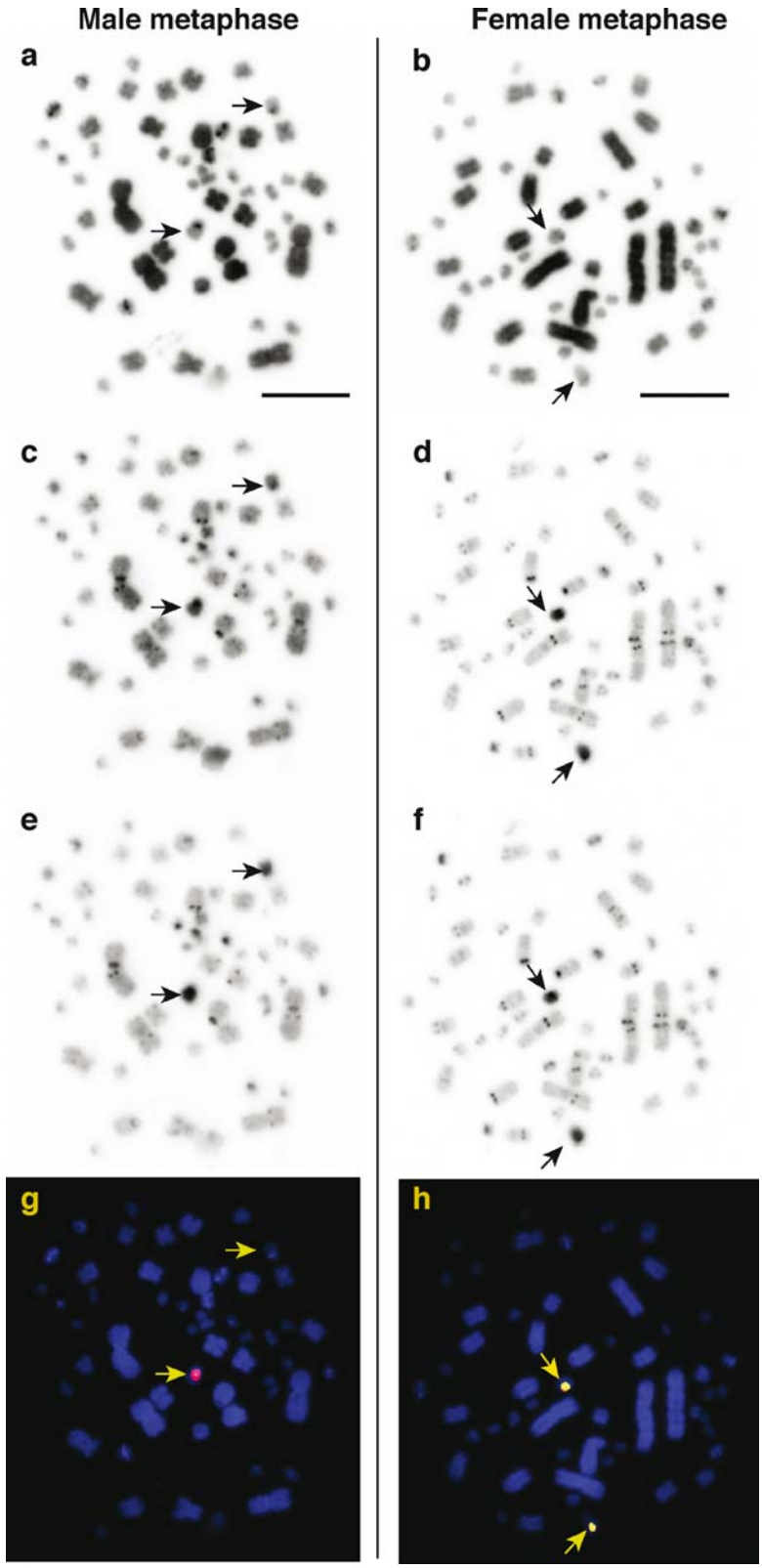

Figure 3. CGH (grey images) in the chromosomes of *Chelodina longicollis* Male (left column) and female (right column). **a**, **b**: DAPI-stained metaphase chromosome spread; **c**, **d**: SpectrumGreen-labelled female total genomic DNA; **e**, **f**: SpectrumRed-labelled male total genomic DNA. Arrows indicate X and Y chromosomes; **g**, **h**: merged images. Scale bar represents 10 μm.

specimen. The fluorescent *in situ* hybridization (FISH) of differentially labelled and co-precipitated male and female total genomic DNA probe produced a sex-specific hybridization pattern in this species (Figure 3). A differential FISH signal was detected in males only, and involved a pair of microchromosomes. One microchromosome in males produced prominent bright hybridization signal with a less intense signal on its homologue, which is virtually unnoticeable in the merged image (Figure 3g). This identified a male-specific Y chromosome and established male heterogamety (XX/XY) in this species. Females showed no difference between the homologues of this microchromosome pair (Figure 3).

Chromosome banding and staining

The chromosomes of two males and one female were investigated by C-banding and 45 cells were analysed from each individual. Small centromeric bands were observed in most of the microchromosomes in both of the males and the female but were not very prominent in macrochromosomes (Figure 4a, b).

In addition, C-banding identified a pair of highly heterochromatic microchromosomes in both males and the female. The distribution of C-banding material (constitutive heterochromatin) was different in the males and the female. In the female, both members of the microchromosome pair had only one central band of constitutive heterochromatin, identifying the X. In the males, one member of this pair had the same central band, but the other had two large blocks of constitutive heterochromatin of unequal size, identifying the Y chromosome. We were able to resolve the difference in C-banding between the X and Y chromosomes in ~70% of the male metaphase spreads analysed, but it was not resolvable in rest of the ~30% cells in which the short chromosomes were too contracted.

Sex differences in GTG-banding in *C. longicollis* were also sought in one female and one male, and 50 cells were analysed from each turtle (Figure 4c, d). GTG bands were present in all macrochromosomes and at least one band was present in all microchromosomes. Comparison of the GTG-banded karyotypes from the male and the female revealed no morphological differentiation between the X and Y microchromosomes (Figure 4c, d). A series of experiments involving a trypsin treatment with varying duration of Giemsa staining still failed to identify sex-specific GTG-banding patterns (T. Ezaz, data not shown).

Although late replication banding on macrochromosomes produced a good replication pattern, we did not observe any replication asynchrony on the X or Y microchromosome in either sex (Figure 4e, f).

Reverse fluorescence staining using CA3 also revealed a clearly different banding pattern on the X and Y microchromosomes (Figure 5). Again one male and one female were tested and 40 cells were analysed for each individual. This staining technique produced a single central band on the X microchromosomes and a large band, covering most of the q-arm, on the Y (Figure 5).

The X and Y chromosomes detected by CGH, C-banding and CA3 staining were very faint when stained with DAPI, indicating that they were AT-poor in sequence, like most of the microchromosomes of *C. longicollis* (Figure 1). The bright CA3 staining confirmed that these microchromosomes were indeed GC-rich.

Superimposing DAPI and CA3 stained images revealed the shapes of these DAPI-faint microchromosomes (Figure 5). For example, the strong DAPI bands in the centromeric regions of the X and Y microchromosomes make them appear small, but CA3 staining reveals much longer arms, placing the X and Y within the larger microchromosomes (Figure 5). This makes them much easier to identify.

Discussion

The first chromosomal studies of reptiles were in the 1920s, but it was not until the 1960s that sex chromosome heteromorphy was conclusively demonstrated in reptiles (reviewed in Gorman 1973).

The search for heteromorphic sex chromosomes is a crucial initial step in the investigation of the sex determining system for any taxon (Valenzuela *et al.* 2003). Importantly, the establishment of heteromorphic sex chromosomes has significant fitness consequences related to the loss of genes from the heterogametic chromosome by drift (Muller's ratchet), genetic hitchhiking, intralocus conflict for sexually selected genes, biased content of fertility genes or cognitive function genes, and sexual dimorphism (Charlesworth *et al.* 2005, Balaresque *et al.* 2004,

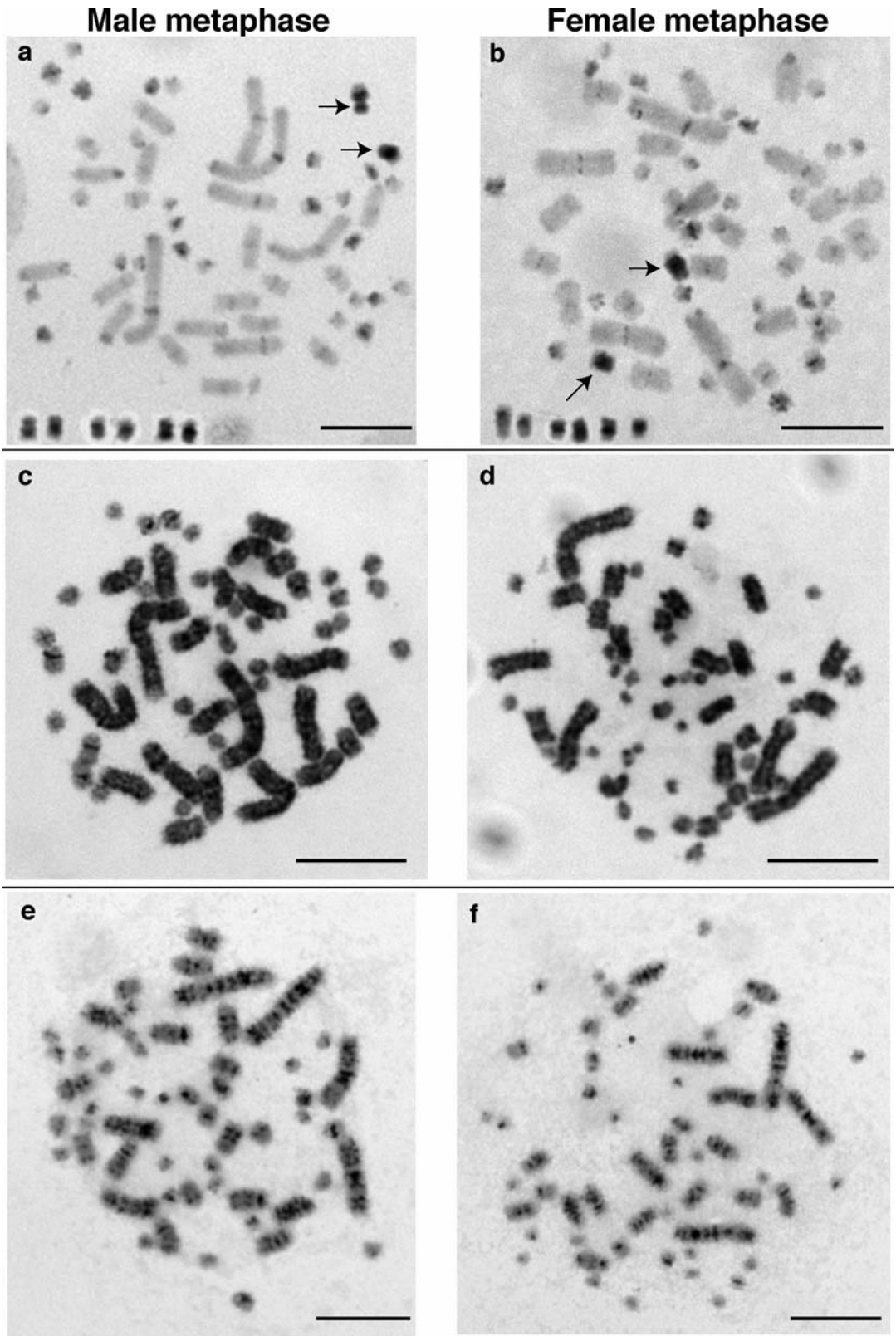

Figure 4. C-banding, GTG-banding and replication banding in *Chelodina longicollis* male (left column) and female (right column). **a**, **b**: C-banding; arrows indicate X and Y chromosomes; three more sex chromosome pairs are also in insets; **c**, **d**: GTG-banding; **e**, **f**: replication banding. Arrows indicate X and Y chromosomes. Scale bar represents 10 μm.

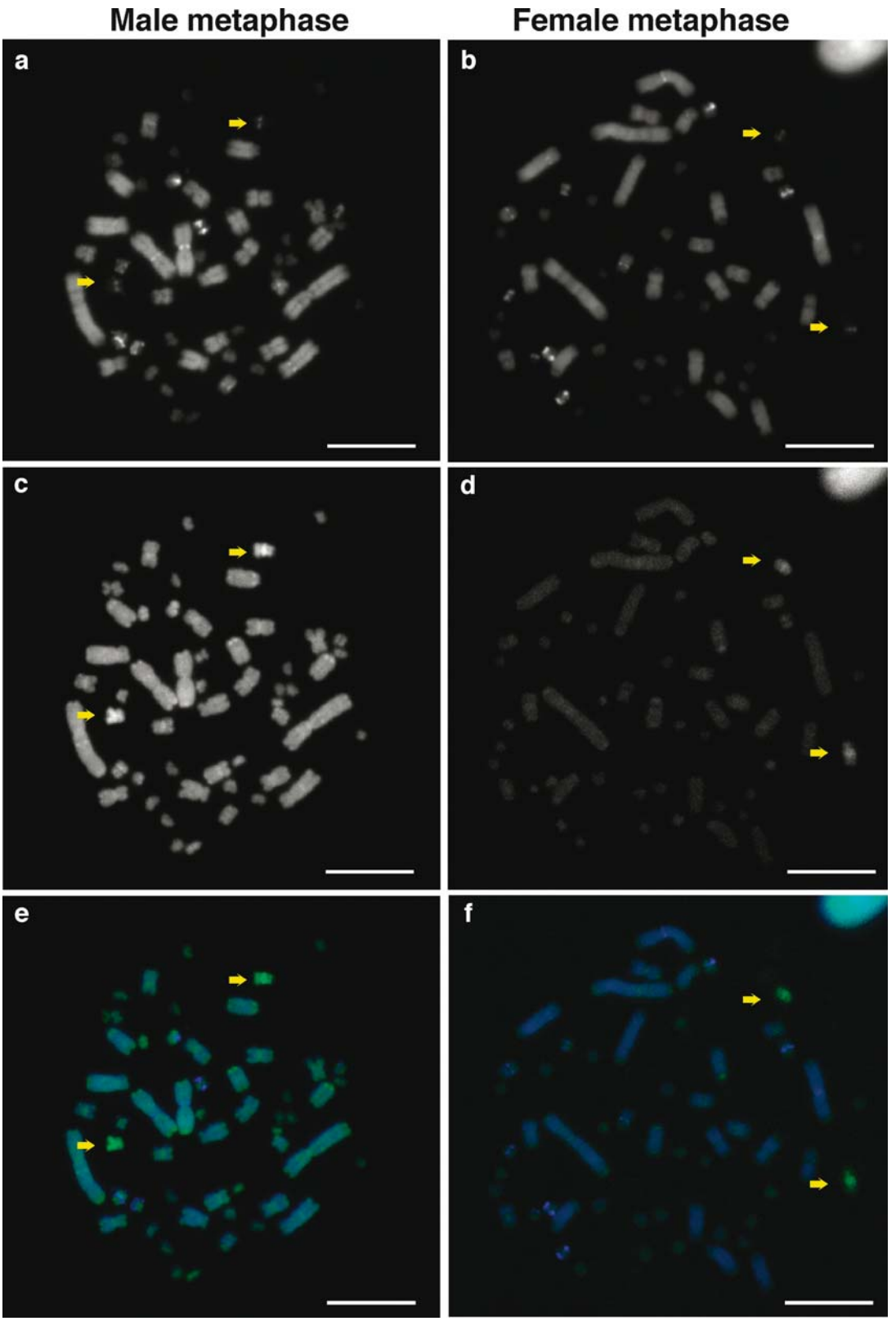

Figure 5. Reverse fluorescence banding (DAPI/Chromomycin A3) in *Chelodina longicollis* male (left column) and female (right column). **a**, **b**: DAPI-stained; **c**, **d**: CA3-stained male; **e**, **f**: DAPI/CA3-stained. Arrows indicate X and Y chromosomes. Scale bar represents 10 μm.

Fitzpatrick 2004, Lindholm *et al*. 2004, Graves *et al*. 2002, Rice 1984). It is essential to detect the existence of sex chromosomes in order to understand the evolutionary dynamics of traits they bear (e.g., sexual dimorphisms; Rice 1984). In addition the identification of the heterogametic sex (XY or ZW system) may help explain species differences in evolution through sexual selection (Reeve and Pfenning 2003).

The karyotype of *Chelodina longicollis* (2n = 54) was first reported to consist of 11 pairs of macrochromosomes and 16 pairs of microchromosomes, based on Giemsa staining (Bull & Legler, 1980). Our investigation confirmed the diploid complement of 2n = 54, but we have designated 12 pairs as macrochromosomes and 15 pairs as microchromosomes, based on DAPI staining; the discrepancy is due to assignment of one pair as a macro- rather than a microchromosome on the basis of a range of staining techniques. The size transition from macro- to microchromosomes is more gradual in *C. longicollis* than the sharp demarcation typical of many snakes and lizards. In our study, DAPI staining indicates that the two size classes also have distinct staining intensity; macrochromosomes are darkly stained with DAPI, whereas microchromosomes are lightly stained (Figure 1).

In our present study of *C. longicollis*, distinguishable sex chromosomes were consistently detected by CGH, as well as by C-banding and reverse fluorescence staining in most metaphase spreads but not by GTG- and replication banding. Unlike the dragon lizard *P. vitticeps* (Ezaz *et al*. 2005), the cryptic sex microchromosomes in *C. longicollis* are heterogametic in the male sex (XX/XY sex microchromosomes). It is unlikely that the *C. longicollis* sex chromosomes could have been detected by traditional approaches alone. The GTG-banding and late replication banding did not distinguish the X and Y chromosome, and C-banding and the reverse fluorescence banding on their own were not sufficiently clear to be conclusive in this species, unlike *P. vitticeps*.

Our experiments suggest that heterochromatin has accumulated in both the sex chromosomes of this species, and also indicated its probable sequence composition, which is different in the X and Y (Figure 5). Comparative genome hybridization (CGH) and C-banding differentiated the Y from the X chromosome but neither technique could reveal the nature of the sequences responsible for the heteromorphism. The long arm of the Y chromosome was completely hybridized by CA3, indicating that it is GC-rich, but the short arm was DAPI-bright and CA3-negative, indicating that it is AT-rich. Many other GSD species with apparently homomorphic chromosomes may have cryptic sex chromosomes that can be revealed by differential staining techniques (see also Ezaz *et al*. 2005).

The difference between the X and Y in *C. longicollis* is rather subtle, suggesting they are at an early stage of sex chromosome differentiation and there has been insufficient time since the origin of the proto-sex chromosomes for large-scale differences to have accumulated. This hypothesis is consistent with the idea that TSD is the ancestral sex-determining mechanism in turtles and that GSD has independently evolved many times within Testudines (Olmo 1986, Janzen & Krenz 2004). The most recent evolutionary transition from TSD to GSD in the ancestral lineage of *C. longicollis* might therefore have occurred relatively recently. Alternatively, sex chromosome homomorphy could be a stable state as it is in the ancient sex-determining systems of some snakes and amphibians (Solari 1994, Miura 1995).

Our findings suggest that sex-specific heterochromatinization involving a pair of microchromosomes was an early event in differentiation of sex chromosomes in *C. longicollis*. Heterochromatin accumulation is believed to be an early step in sex chromosomal differentiation in some primitive snakes and lizard species (Olmo *et al*. 1984, Ray-Chaudhury *et al*. 1971, Singh *et al*. 1976). The difference in chromosomal distribution of heterochromatin between X and Y chromosomes could also be the result of an intrachromosomal rearrangement in the Y chromosome. However, further study involving heterochromatin as well as chromosome-specific probes would be needed to demonstrate such rearrangements.

Although sex microchromosomes are quite common in some snakes and lizard species (for detail see Donnellan 1985, Ezaz *et al*. 2005, Olmo & Signorino 2005), this is the first demonstration of sex microchromosomes in a turtle. Microchromosomes may therefore be involved in reptilian sex determination more commonly than thought previously. It is possible that chromosome rearrangements involving microchromosomes may play a major role in sex-chromosomal differentiation in reptilian lineages. It will therefore be worthwhile to examine more closely

the organization and evolution of sequences of such sex microchromosomes. This may lead to the discovery of novel genes in the sex determination pathway in turtles as well as other species in the reptilian lineages.

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