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# The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes

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## Abstract

The bearded dragon, *Pogona vitticeps* (Agamidae: Reptilia), is an agamid lizard endemic to Australia. Like crocodilians and many turtles, temperature-dependent sex determination (TSD) is common in agamid lizards, although many species have genotypic sex determination (GSD). *P. vitticeps* is reported to have GSD, but no detectable sex chromosomes. Here we used molecular cytogenetic and differential banding techniques to reveal sex chromosomes in this species. Comparative genomic hybridization (CGH), GTG- and C-banding identified a highly heterochromatic microchromosome specific to females, demonstrating female heterogamety (ZZ/ZW) in this species. We isolated the *P. vitticeps* W chromosome by microdissection, re-amplified the DNA and used it to paint the W. No unpaired bivalents were detected in male synaptonemal complexes at meiotic pachytene, confirming male homogamety. We conclude that *P. vitticeps* has differentiated, previously unidentifiable W and Z micro-sex chromosomes, the first to be demonstrated in an agamid lizard. Our finding implies that heterochromatinization of the heterogametic chromosome occurred during sex chromosome differentiation in this species, as is the case in some lizards and many snakes, as well as in birds and mammals. Many GSD reptiles with cryptic sex chromosomes may also prove to have micro-sex chromosomes. Reptile microchromosomes, long dismissed as non-functional minutiae and often omitted from karyotypes, therefore deserve closer scrutiny with new and more sensitive techniques.

## Introduction

Reptiles are typically classified as exhibiting one of two alternative sex-determining mechanisms: genotypic sex determination (GSD) or temperature-dependent sex determination (TSD). In GSD, offspring sex is determined by the genes inherited from the parents, with little or no influence of environmental factors, and is the sex-determining mechanism found in all birds and mammals, as well as most fish and amphibians. In TSD, sex is determined by the incubation temperature prevailing during embryonic development. Despite the traditional classification of reptiles into one of these two modes of sex determination, in some species there may be some interaction between genotype and thermal environment in determining sex (Shine *et al*. 2002, Sarre *et al*. 2004).

All GSD species have sex chromosomes, which are by definition the chromosome pair (or pairs) that house the sex-determining gene(s). The homologues of a sex chromosome pair are differentiated at some level in one of the sexes, called the heterogametic sex. Mammals exhibit male heterogamety (XX female, XY male) whereas birds have female heterogamety (ZZ male, ZW female). Both systems have been reported in GSD reptiles. In contrast to mammals and birds, there is great variability in the degree of differentiation between the sex chromosome homologues in reptiles. In many snakes and lizards, the heterogametic Y or W chromosome is highly differentiated in both morphology and sequence composition, as in avian and mammalian sex chromosomes. In particular, heterochromatinization of one sex chromosome varies greatly in GSD snakes and lizards, ranging from a small block of heterochromatin to the entire chromosome. Chromosomal rearrangements such as centric fusions, pericentric inversions and translocations have also contributed to sex chromosomal differentiation in lizards (for review see Olmo 1986). These associated differences in chromosome morphology (heteromorphy), detected by cytological techniques, have been the primary method of establishing the presence and type of heterogamety in reptiles.

Heteromorphic sex chromosomes are common in snakes, frequent in lizards, and rare in turtles. In other lizards and most turtles, however, sex chromosomes are not detectable by traditional cytological techniques (for review see Olmo 1986, Janzen & Paukstis 1991). If TSD has been excluded as the sex-determining mechanism through incubation temperature experiments, then these species are presumed to be GSD species with homomorphic sex chromosomes, indistinguishable by differences in size, morphology or heterochromatinization. In some instances, the difference between the sex homologues is so subtle that a high-resolution cytogenetic technique is required to identify the sex chromosome pair.

Lizards (Order Squamata, Suborder Sauria), with around 4765 extant species (Uetz 2005), have the most diverse array of sex-determining mechanisms and sex chromosome systems of any reptile group. TSD, GSD and parthenogenetic species are well documented among lizards. Karyotypes have been described for around 20% of species across the 18 families. Sex chromosomes have been described in 18% of those species karyotyped, representing ten of the 18 families. Both male (XX/XY) and female (ZZ/ZW) heterogamety and XY- and ZW-derived multiple chromosomal sex-determining systems (e.g., $X_1X_1X_2X_2/X_1X_2Y$ and $Z_1Z_1Z_2Z_2/Z_1Z_2W$) have been described in several families (Olmo 1986, Janzen & Paukstis 1991, Olmo & Signorino 2005). Sex chromosomes have been observed in a minority (169 of 939) of karyotyped species, mostly in the families Iguanidae and Lacertidae (Olmo 1986, Janzen & Paukstis 1991, Olmo & Signorino 2005).

Dragon lizards (Family Agamidae) comprise ~380 species (Uetz 2005) widespread across the world. There are 70 species currently described in Australia (Cogger 1996). Karyotypically, agamids are a reasonably well-studied group with karyotypes available for 27% (104/380) of species described worldwide, including 31% (22/70) of Australian species (Witten 1983, Janzen & Paukstis 1991, Olmo & Signorino 2005). Morphologically differentiated sex chromosomes have been described in only one species of agamid lizard, *Phrynocephalus vlangalii* (Zeng *et al.* 1997). In that species, the sex chromosomes are the largest macrochromosome pair and show female heterogamety (ZZ/ZW). Both GSD and TSD have been described in Australian agamids, and show a somewhat haphazard distribution amongst species (Harlow 2001). In some cases, these alternative sex-determining mechanisms are exhibited by sister species within the same genus, suggesting recent evolutionary transitions between TSD and GSD. The Australian Agamidae therefore represents an excellent group for investigations of the evolution of sex-determining mechanisms in reptiles.

The genus *Pogona* (bearded dragons) comprises seven species, all endemic to Australia. The central bearded dragon, *P. vitticeps*, the most common agamid in the global pet trade, is distributed from the semi-arid to arid interior of New South Wales, Victoria, and Queensland to the eastern half of South Australia and the Northern Territory. It reaches 33–61 cm in total length, and colour ranges from dull brown to tan with red or gold highlights. The sex ratio of *P. vitticeps* does not vary significantly from 1:1 over a range of constant incubation temperatures (Viets *et al.* 1994, Harlow 2001), indicating that this species has genotypically determined sex, and is not a TSD species. Witten (1983) first described the Giemsa-stained karyotypes of two species of bearded dragon, *P. vitticeps* and *P. barbata*, both with a diploid chromosome complement of 2n = 32 (12

macro- and 20 microchromosomes), but did not identify sex chromosomes.

Here, we report on our use of molecular cytogenetic techniques to identify the cryptic sex chromosomes of *P. vitticeps*. We applied comparative genomic hybridization (CGH) along with differential banding procedures (C- and GTG-banding). CGH is a molecular cytogenetic technique that allows detection of DNA sequence copy number changes throughout the genome in a single hybridization. The sensitivity of CGH in detecting gains or losses of DNA sequences is approximately 2–20 Mb. CGH was originally developed to detect molecular differences between normal and cancer cell genomes at the cytogenetic level (Kallioniemi *et al.* 1992). However, this technique has successfully been adapted to demonstrate the sex chromosomal differences in a diverse group of animals with varying degrees of sex chromosomal differences (Traut *et al.* 1999, Barzotti *et al.* 2000, Traut *et al.* 2001). We also describe the isolation and development of a W chromosome-specific FISH paint by microdissection, and use of this paint to further confirm the female-specific nature of that microchromosome.

## Materials and methods

### *Animals*

Central bearded dragons (*Pogona vitticeps*) were collected from the arid outback country in north-western New South Wales (NSW) and south-west Queensland (QLD), in a roughly rectangular region with corners at Bourke, Hungerford (NSW), Adavale and Charleville (QLD), Australia. Dragons were kept in captivity at the University of Canberra until required for fresh blood or tissue collection for culturing. For short-term culture, blood was collected from the caudal vein with a heparinized (Heparin Sigma) 25-gauge needle attached to a 1–2-ml disposable syringe. Prior to tissue collection for fibroblast culture, animals were euthanized by intracranial injection of Nembutal (Sigma). Pericardial and corneal tissues were collected for fibroblast culture. Euthanized animals were sexed by dissection of the gonads. Non-euthanized animals from which blood samples were taken were all females known to have been previously gravid.

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 issued by the State Governments (Queensland animal collection permit no. WISP01040203, New South Wales animal collection permit no. S10661), and under 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). The number of individuals and the number of cells used in the various experiments described throughout this publication are summarized in Table 1.

### *Metaphase chromosome preparation*

Metaphase chromosome spreads of *P. vitticeps* were prepared from short-term culture of whole blood or peripheral blood leukocytes, and also from fibroblast cell lines. Approximately 100 µl blood was used to set up 2 ml culture in Dulbecco's Modified Eagle's

*Table 1.* Number of individuals and cells examined in this study.

| | Number of males | | Number of females | |
|---|---|---|---|---|
| Experiment | Number of individuals | Number of cells | Number of individuals | Number of cells |
| Karyotyping | 2 | 80 | 2 | 80 |
| Meiosis (light microscopy) | 1 | 25 | 0 | 0 |
| Meiosis (electron microscopy) | 1 | 40 | 0 | 0 |
| CGH | 3 | 90 | 3 | 90 |
| C-banding | 6 | 180 | 6 | 180 |
| GTG-banding | 2 | 60 | 2 | 60 |
| Replication banding | 2 | 40 | 2 | 40 |
| Reverse fluorescent banding | 2 | 50 | 2 | 60 |
| W microdissection | 0 | 0 | 3 | 30 |

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 4 h 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 & 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.

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.

*Meiotic chromosome preparation*

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 as described earlier.

*Preparation of synaptonemal complex (SC) spreads*

SC preparation was performed in male *P. vitticeps* following the protocol described by Harvey *et al.* (2002) with minor modifications. Briefly, the testes were minced in a small Petri dish containing Hanks' saline solution (Sigma), and a cell suspension was made in 1.5 ml Hanks' solution. The cell suspension was pelleted by centrifugation (100 *g* for 2 min), resuspended in 0.2 mol/L sucrose, 0.2% SDS (each buffered to pH 8.5 with 0.01 mol/L sodium tetraborate), and fixed with 80 μl of 4% paraformaldehyde (buffered to pH 8.5 with 0.2 mol/L sodium tetraborate).

Glass slides were cleaned with ethanol and coated with plastic solution (0.5% w/v, broken 50-ml Falcon tube in chloroform), then rinsed in 0.4% Photoflo solution (Kodak). Approximately 100–250 μl of cell suspension was gently pipetted onto a plastic-coated slide and air-dried horizontally for 4 h in a fume hood. Air-dried slides were rinsed for 1 min in Photoflo solution, air-dried, and stained with 50% silver nitrate as described by Howell & Black (1980). After localization and marking under a light microscope, the pachytene cells were transferred onto 50-mesh electron microscope copper grids (Agar Scientific) following the standard water floating technique, and examined at 80 kV using a Philips 301 transmission electron microscope. The SC spreads were analysed using Image Pro Plus 3.0 software.

*DNA extraction and labelling*

Total genomic DNA was extracted from whole blood following the protocol of Ezaz *et al.* (2004). Female total genomic DNA was labelled with SpectrumGreen-dUTP (Vysis, Inc.) while male total genomic DNA was labelled with SpectrumRed-dUTP (Vysis, Inc.) by nick translation.

*Comparative genomic hybridization (CGH)*

We followed the procedure of comparative genomic hybridization described by Traut *et al.* (1999) with modifications. Slides were denatured for 2–2.5 min at 70°C in 70% formamide, 2×SSC, dehydrated through an ethanol series, air-dried and kept at 37°C until probe hybridization. For each slide (one drop of cell solution), 250–500 ng of SpectrumGreen-labelled female and SpectrumRed-labelled male DNA was coprecipitated 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 alternately male and female DNA as competitor.

The coprecipitated probe DNA was resuspended in 20 µl hybridization buffer (50% formamide, 10% dextran sulfate, 2×SSC, 40 mmol/L sodium phosphate pH 7.0 and 1× Denhardt's solution). The hybridization mixture was denatured at 70°C for 10 min, 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 days. Slides were washed once at 60 ± 1°C in 0.4×SSC, 0.3% Tween 20 for 2 min followed by another wash at room temperature in 2×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 colocalize the source images into a colour image.

*Chromosome banding (GTG-banding, C-banding and replication banding)*

GTG-banding was performed following the techniques described in ISCN (1985). Freshly dropped or up to 10-day-old slides aged at 37°C were treated with 0.05% trypsin (Gibco BRL) solution (in 1× Dulbecco's PBS-CMF) for 15–60 s. Slides were 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. Slides were rinsed in distilled water, air-dried and then mounted with D.P.X. (Ajax Chemicals) neutral mounting medium.

C-banding was performed as described by Sumner (1972) with slight modification. Slides were aged at room temperature for 2–3 days, soaked in 0.2 N 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 2×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. Slides were rinsed in distilled water, air-dried, and mounted with D.P.X. (Ajax Chemicals) neutral mounting medium.

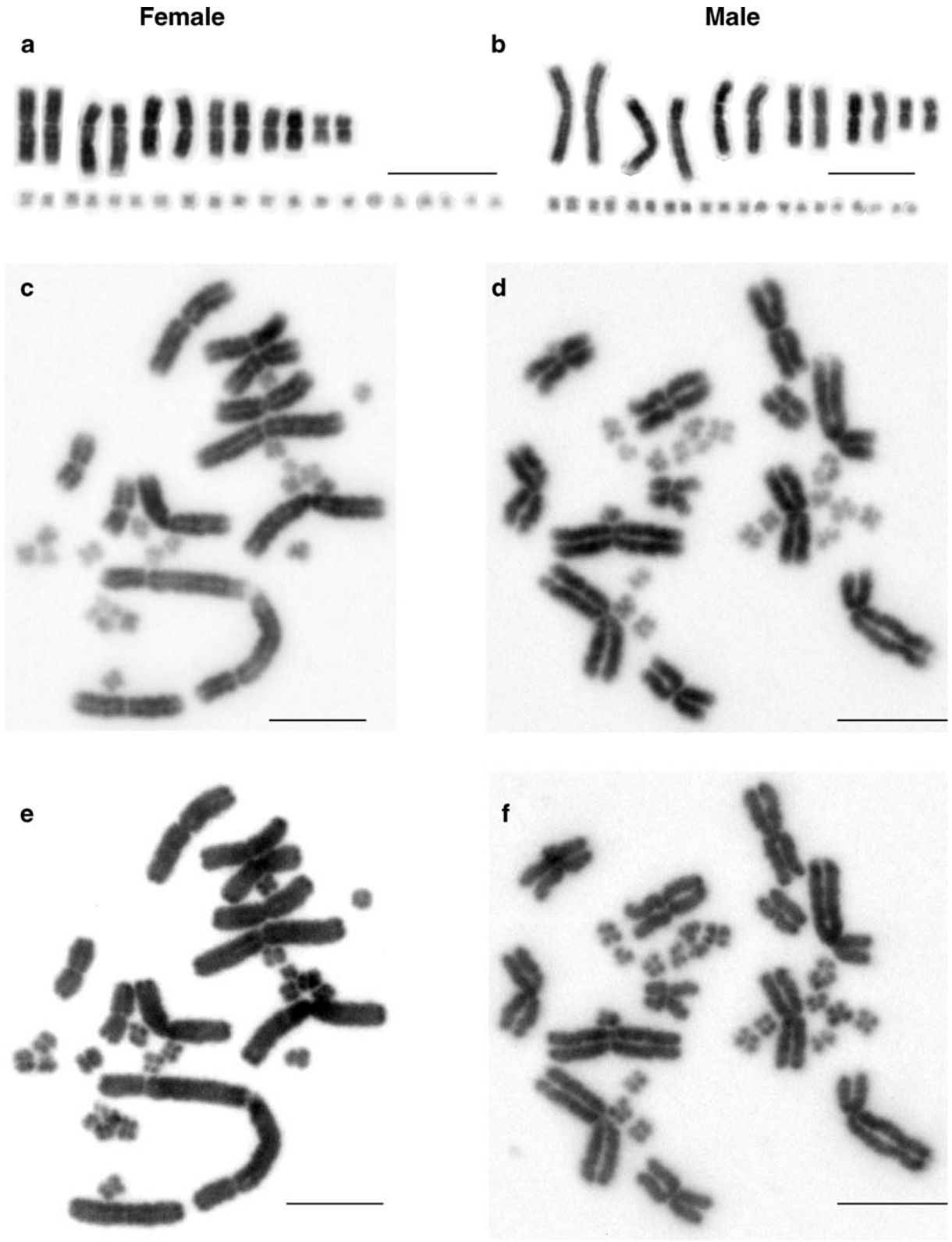
Late replication banding was performed as described by Miura (1995). BrdU incorporated chromosome preparations were dropped onto microscope slides as described above. The slides were then incubated overnight at 55°C, immersed in methanol for several seconds and incubated for 3–5 min at 40°C in tetrasodium EDTA–Giemsa solution (3% Giemsa solution in 2% tetrasodium EDTA).

*Chromosome microdissection*

Fresh metaphase cell suspension from female *P. vitticeps* was dropped onto cover slips, air-dried and GTG-banded. A single W chromosome, recognized by GTG-banding, was microdissected under a Zeiss Axiovert microscope using a sterile (UV-irradiated) glass needle. Movement of the glass needle was controlled with a three-dimensional Eppendorf micromanipulator. The needle bearing the isolated W was broken into a 0.2-ml PCR tube with 5 µl of collection drop (containing 50 mmol/L Tris-HCl, pH 7.5), 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 0.1 mmol/L DTT, 30 µg/ml BSA). To each tube, 1.5 units of topoisomerase (Invitrogen) was added and mixed well, followed by brief centrifugation and incubation of the tubes at 37°C for 30 min. The topoisomerase was then inactivated by heating the tubes at 95°C for 10 min.

The microdissected W chromosome DNA was then amplified by DOP-PCR (Telenius *et al*. 1992). Fifty mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0), 2.5 mmol/L $MgCl_2$, 200 µmol/L each dNTP, 2 µmol/L degenerate oligonucleotide primer (DOP 6-MW primer: 5′-CCG ACT CGA GNN NNN NAT GTG G-3′) and 1.75 units of Taq DNA polymerase (Promega) were added to the 5 µl of topoisomerase-treated microdissected chromosome DNA solution and PCR amplified (8 cycles of 95°C for 1 min, 30°C for 1 min and 37°C for 3 min, and 35 cycles of 30°C for 2 min 20 s, 95°C for 1 min, 56°C for 1 min followed by 72°C for 2 min). From this first-round DOP-PCR product, 1–2 µl was used as a template for a secondary DOP-PCR incorporating digoxigenin-11-dUTP or biotin-16-dUTP (Roche) in a 35-cycle moderately stringent PCR (95°C for 1 min, 56°C for 1 min followed by 72°C for 2 min).

Fluorescent *in-situ* hybridization was performed as previously described (Pinkel *et al*. 1988, Yang *et al*. 1995) with modifications. Briefly, 1.5 µl of


Female
Male
a
b
c
d
e
f

labelled PCR product, 14.5 µl of hybridization mixture (50% deionized formamide, 10% dextran sulfate, 2×SSC, 40 mmol/L sodium phosphate ($Na_2HPO_4$ and $NaH_2PO_4$) pH 7.0, 1× Denhardt's solution), 10–20 µg of boiled genomic DNA (as a competitor), and 20 µg of glycogen (as carrier) were denatured at 70°C for 10 min. The repetitive DNA was pre-annealed for 30 min at 37°C. The slides with metaphase chromosomes were denatured at 70°C for 2 min in 70% formamide, 2×SSC. The denatured probe was dropped onto a denatured slide, covered with a 22 × 32-mm cover slip and hybridization was performed at 37°C in a moist chamber for 24–48 h.

After hybridization, the slides were washed twice for 5 min in 50% formamide, 2×SSC (pH 7.0) at 42°C, twice for 5 min in 0.5×SSC (pH 7.0) at 42°C, and blocked with 4×SSC (pH 7.0), 3% BSA, 0.05% Tween 20 at 37°C for 30 min. Probe was detected with Cyanin 3 (Cy3)-conjugated anti-digoxigenin antibody. Chromosomes were counterstained with 1 µg/ml DAPI in 2×SSC for 1 min. Images were captured using a Zeiss Axioplan epifluorescence microscope as described above.

### *Terminology*

Terminology used to describe sex chromosome differentiation has been made difficult by the advent of fine-scale molecular approaches. We use the following terminology. Sex chromosomes carry the sex-determining genes. They are differentiated at some level but this differentiation may be at the level of genes and not at the cytological level. Homomorphic sex chromosomes are differentiated but not at the level that can be detected cytologically as gross morphology differences or with C- or GTG-banding under a light microscope. Heteromorphic sex chromosomes are differentiated at the level that can be detected cytologically as gross morphology differences or with C- or GTG-banding under a light microscope. Cryptic sex chromosomes is a term used in the literature for sex chromosomes whose differentiation has gone unnoticed because of their size or failure to demonstrate differential banding, but which may turn out to be heteromorphic on closer examination (e.g., the cryptic sex chromosomes of *P. vitticeps*, present study).

## Results

### *Karyotypes of* Pogona vitticeps

The DAPI-stained mitotic karyotypes of two females and two males were examined. For each individual, a total of 40 counts was made of mitotic chromosome spread at metaphase (Table 1). Comparison of the karyotypes from males and females did not reveal the presence of any morphologically differentiated sex chromosomes in either sex (Figure 1a, b). The diploid chromosome complement of *P. vitticeps* is 2n = 32 and the karyotype is represented by 12 macrochromosomes and 20 microchromosomes (12 M + 20 m) with a distinct break in size between the macro- and microchromosomes (Figure 1a, b). Among the 12 macrochromosomes, 10 (including the largest pair) are metacentric while the second largest pair is submetacentric. The centromeric positions of the microchromosomes could not be determined accurately because of their small size as well as their weak DAPI staining (Figure 1c, d). Reverse fluorescent staining with DAPI and CA3, which preferentially binds to GC-rich DNA, revealed that the microchromosomes are GC-rich, and most are metacentric (Figure 1e, f). For CA3 staining, 25 and 30 cells were examined respectively from each male and female (Table 1).

The first meiotic division (diakinesis/metaphase I) from *P. vitticeps* testis showed 16 bivalents, also with distinct size dichotomy as observed in mitotic metaphase chromosomes. A total of 25 cells from one male was examined and no univalency was observed (Figure 2a). The synaptonemal complex spreads of *P. vitticeps* at the pachytene stage contained 16 silver-stained bivalents, 6 large and 10 small (Figure 2b). The lateral elements were well differentiated but the central region of the SCs and the kinetochores were not clearly revealed. A total of 40 meiotic pachytene nuclei from one male were analysed and no pairing aberrations were observed;

*Figure 1.* Grey images of DAPI- and chromomycin A3-stained metaphase chromosome karyotypes and spreads from female and male *Pogona vitticeps*. (**a**, **b**) DAPI-stained female and male karyotypes; (**c**, **d**) DAPI-stained female and male metaphase chromosome spread; (**e**, **f**) chromomycin A3-stained female and male metaphase chromosome spread. Scale bars represent 10 µm.

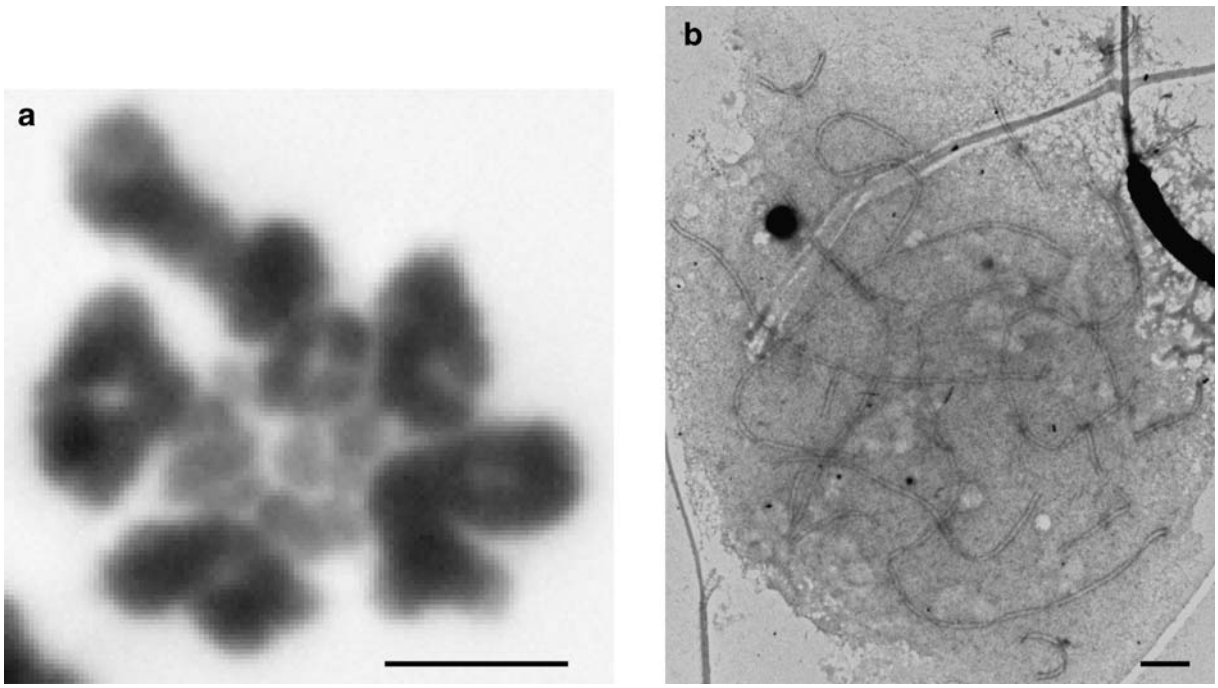



*Figure 2.* Male meiosis in *Pogona vitticeps*. (**a**) Meiotic prophase I; (**b**) synaptonemal complex. Scale bars represent 10 μm.

all homologous chromosomes were fully paired along their entire length (Figure 2b).

*Comparative genomic hybridization (CGH)*

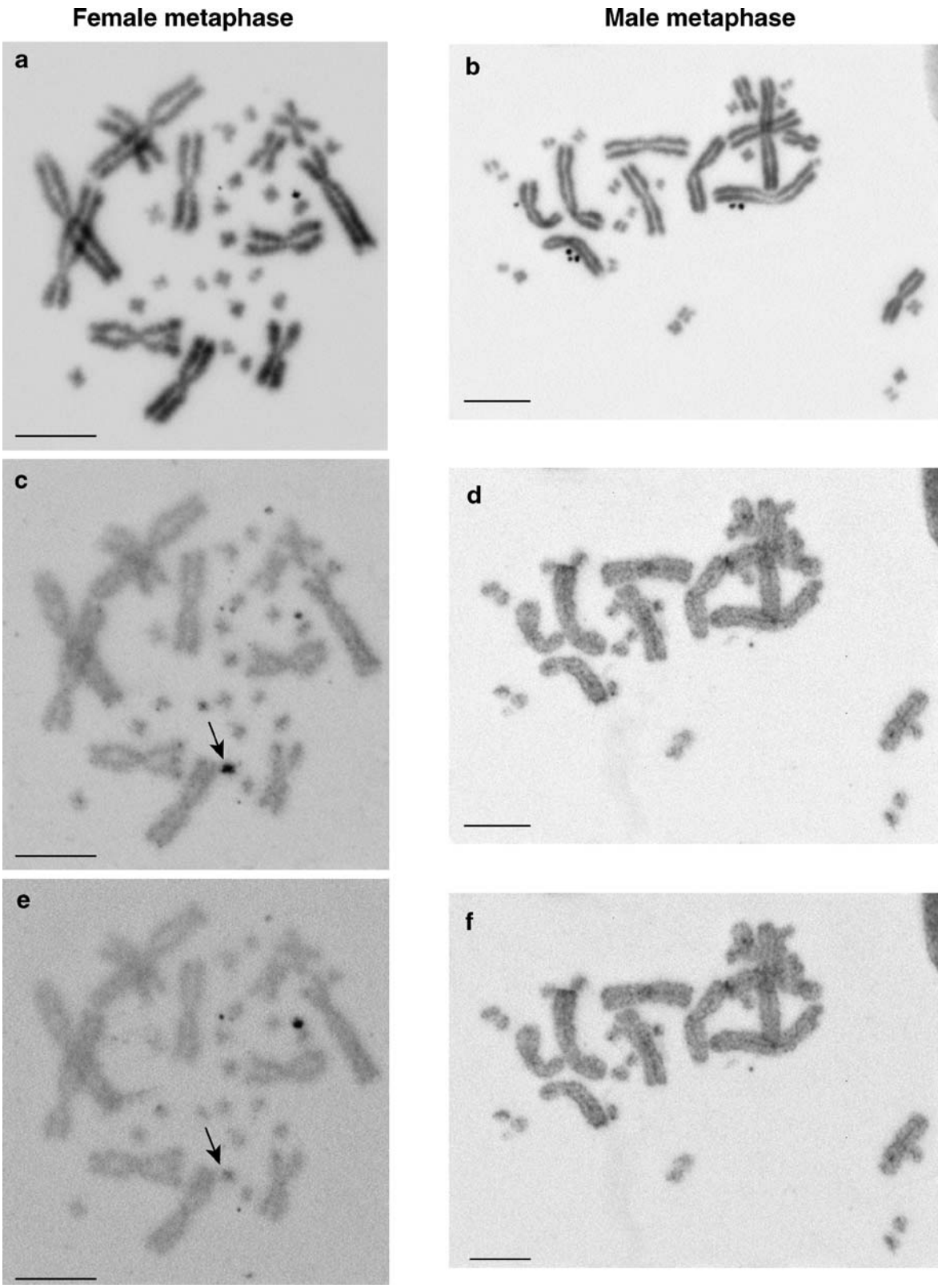
CGH was performed on the chromosomes of three female and three male *P. vitticeps*. Thirty cells were examined from each individual (Table 1). We hybridized the metaphase chromosome preparations with labelled total genomic DNA from a female and differently labelled total genomic DNA from a male. This technique requires the addition of Cot-1 or an excess of boiled denatured unlabelled genomic DNA from the homogametic sex and since we had no *a priori* knowledge of the homogametic sex, reciprocal experiments were performed assuming males or females as the homogametic sex. A very bright hybridization signal was observed in one of the microchromosomes in cells from all females but not on chromosomes from any of the males (Figure 3). This female-specific chromosome is therefore by definition a W chromosome, identifying females as the heterogametic sex. To establish the consistency and to control for any sensitivity differences of the different fluorochromes to different filter sets, male and female genomic DNA were reciprocally labelled and applied to male and female dragon cells. Results were consistent between individuals within each sex (data not shown).

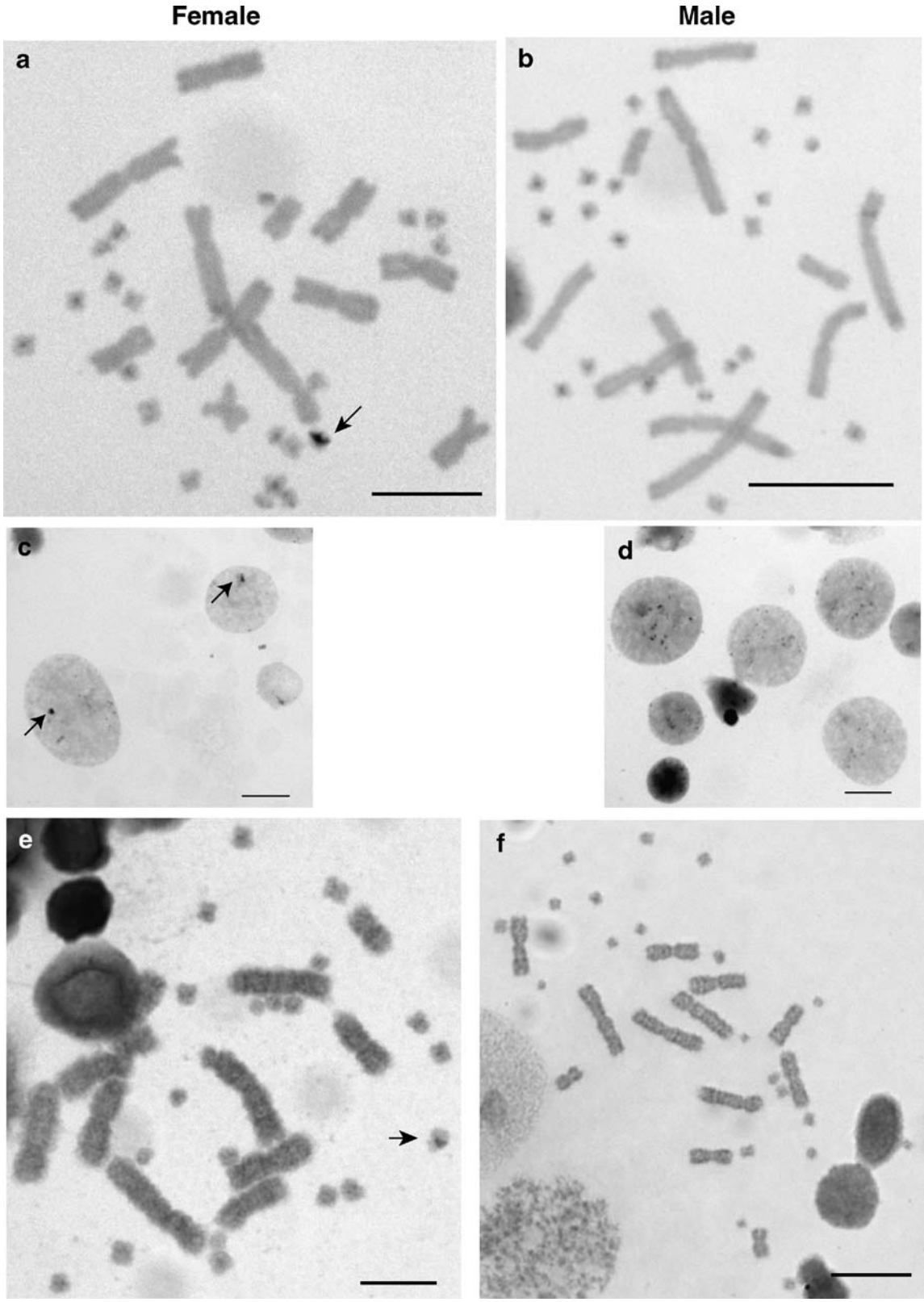
*Chromosome banding*

C-banding (Figure 4a, b) reveals the presence of small centromeric bands in most of the microchromosomes but few of prominence in macrochromosomes. A total of 30 cells from each individual were examined for C-banding (Table 1). A very large constitutive heterochromatic band is present in one of the microchromosomes of all six females but absent in all six males examined (Figure 4a, b). A heavily C-banded body is also seen in interphase nuclei in female but not male cells (Figure 4c, d). This staining, therefore, also identified a highly heterochromatinized W chromosome.

Serial and prominent chromosome banding (e.g., GTG-banding) is often difficult to obtain in fish, amphibians and reptiles. However, prominent GTG-

*Figure 3.* CGH in *Pogona vitticeps* female (left column) and male (right column). (**a**, **b**) DAPI; (**c**, **d**) SpectrumGreen-labelled female total genomic DNA; (**e**, **f**) SpectrumRed-labelled male total genomic DNA. Arrow indicates W chromosome. Scale bars represent 10 μm.


Female metaphase
Male metaphase
a
b
c
d
e
f


Female
Male
a
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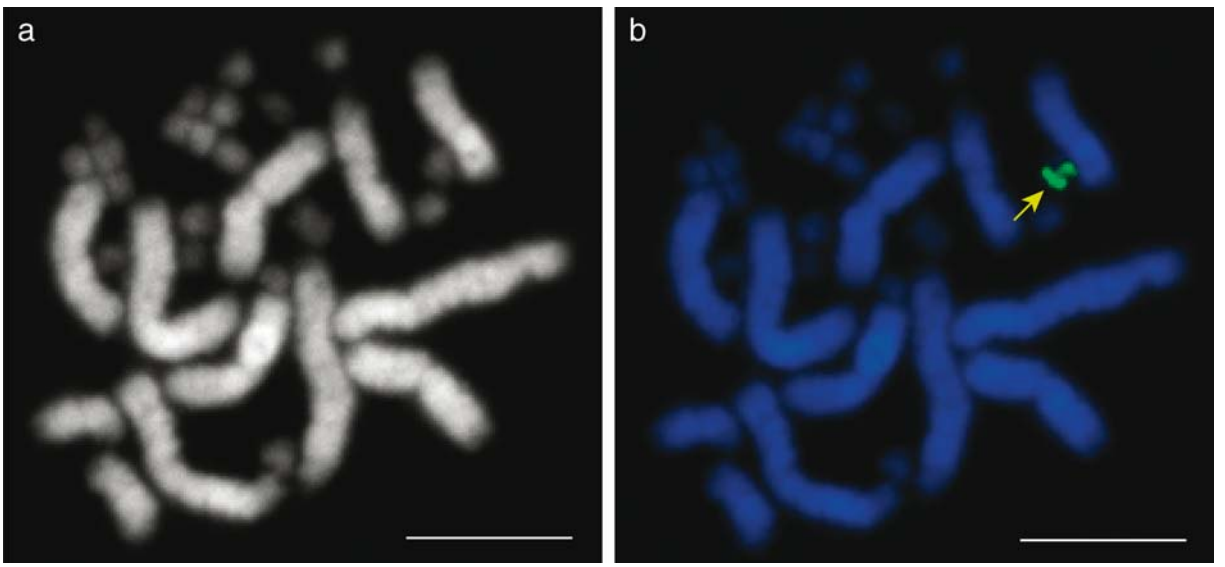


*Figure 5.* FISH localization (grey image) of SpectrumGreen-labelled microdissected W chromosome probe in female metaphase of *Pogona vitticeps*. (**a**) DAPI-stained female metaphase; (**b**) Merged image showing the FISH localization of microdissected W chromosome probe. Arrow indicates W chromosome FISH probe localization on W chromosome. Scale bars represent 10 μm.

bands were reproducible in all macrochromosomes and in most microchromosomes in *P. vitticeps*. In cells from females, but not males, a large band was observed on one microchromosome (Figure 4e, f). Late replication banding also produced prominent banding on macrochromosomes. However, replication timing of the W chromosome was not clear due to insufficient resolution (data not shown). For each individual, 30 cells were examined for GTG-banding and 20 cells were examined for replication banding (Table 1).

Although CGH-, C- and GTG-bandings were consistent in identifying the W chromosome in repeated experiments, none of these methods identified the Z chromosome which was expected to be present in two copies in males and a single copy in females.

### *W chromosome microdissection*

A total of 30 GTG-banded W chromosomes from three females were microdissected and DNA paints prepared. These were used as FISH probes on female and male metaphase spreads. The hybridization of microdissected W chromosome showed signals on only one chromosome in female metaphase spreads (Figure 5) but no signal was detected in male metaphase chromosome spreads (data not shown).

## Discussion

The bearded dragon, *P. vitticeps*, is a common and widespread lizard in its native Australia, and is extremely amenable to captive husbandry. It is therefore an excellent model species for studying reptilian sex determination. Conventionally stained karyotypes of *P. vitticeps* described previously by Witten (1983) showed a diploid chromosome complement of 2n = 32, containing 12 macrochromosomes and 20 microchromosomes, but no heteromorphic sex chromosomes. Our investigation confirmed this diploid karyotype. Previously it was thought that microchromosomes are acrocentric in this species (Witten 1983) but our reverse fluorescence staining demonstrated that most of the microchromosomes are metacentric.

We used molecular cytogenetic techniques combined with differential banding to search for cryptic sex chromosomes in *P. vitticeps*. C- and GTG-bandings have been found to be effective in revealing sex chromosomes in some lizards (Olmo & Signorino

*Figure 4.* Chromosome banding in *Pogona vitticeps*. Female (left column) and male (right column). (**a**, **b**) C-banded; (**c**, **d**) interphase nuclei; (**e**, **f**) GTG-banded. Arrow indicates C- and GTG-banded heterochromatic bands on W chromosome. Scale bars represent 10 μm.

2005). Although CGH has been found to be effective in identifying sex chromosomes in a number of other organisms (Traut *et al.* 1999, Barzotti *et al.* 2000, Traut *et al.* 2001), this is the first application of CGH to identify sex chromosomes in a reptile species.

Our experiments involving CGH-, C- and GTG-banding identified a highly heterochromatinized microchromosome specific to females, thus identifying the W chromosome of a ZZ/ZW sex chromosome system in *P. vitticeps*. Our failure to identify any unpaired chromosomes at meiosis supports this view. Although our experiments failed to identify the Z chromosome cytologically, the identical chromosome counts in male and female mean that that one chromosome must be present in two copies in males and a single copy in females. The identical count in males and females also rules out a multiple sex chromosome system such as those described in lacertid lizards by Olmo *et al.* (1987). The Z chromosome must be a microchromosome, since all the macrochromosomes formed homomorphic homologous pairs at mitosis and heteromorphism of any of the macrochromosomes would have been very obvious, particularly by C- and GTG-banding. The Z chromosome did not appear to contain any substantial regions of GC-rich heterochromatin, although a faint signal on the Z might have been suppressed in preparations containing the large block of GC rich heterochromatin on the W chromosome. The lack of hybridization of the microdissected W probe to any other chromosome in either male or female spreads also suggests that the Z and W chromosomes share few or no sequences. A somewhat similar situation has been reported in two lacertid lizard species: *Takydromus sexlineatus* and *Galloita galloti*, in which the W and Z are otherwise homomorphic, but the W is completely C-banded (Olmo *et al.* 1984). This is the first identification of sex chromosomes in an Australian agamid species, and the first report of micro sex chromosomes in any agamid species. The single other agamid species reported to have heteromorphic sex chromosomes, *Phrynocephalus vlangalii*, also has female heterogamety but, in that case, the largest macrochromosome pair was involved (Zeng *et al.* 1997).

One of the few reported cases of reptile sex chromosomes falling into the microchromosome size range is the multiple sex chromosome system ($X_1X_1X_2X_2/X_1X_2Y$) in the family Iguanidae (Gorman 1973). The W chromosome is also a microchromosome in several species of the family Lacertidae but it has been presumed that these microchromosomes were derived by degradation and deletion of a macrochromosome (Olmo *et al.* 1987). For example, a W microchromosome identified in *Lacerta lepida* by Olmo *et al.* (1987) was heterochromatic and strongly DAPI positive, and therefore AT rich. In *P. vitticeps*, however, all microchromosomes including the W chromosome stained weakly with DAPI, indicating an AT-poor sequence composition. The high GC content of the microchromosomes was confirmed by our reverse fluorescence staining experiment using DAPI and CA3.

The apparently complete differentiation of the heterochromatic and heavily C-banded W chromosome from the Z chromosome in *P. vitticeps* gives some clues to the age of the sex chromosome system in *P. vitticeps*. Heterochromatinization is thought to be an early change that initiated sex chromosomal differentiation in some snake groups (Ray-Chaudhury *et al.* 1971, Singh *et al.* 1976, Olmo *et al.* 1984). On the other hand, the complete differentiation of the W and Z chromosomes and the absence of any clear sequence homology suggests a more ancient and stable sex chromosomal system as observed in snakes and birds. It has been proposed that sex chromosome evolution in lizards and snakes has sometimes followed a parallel pathway (Olmo 1986). Our results suggest that the accumulation of heterochromatin is a significant factor in the differentiation of sex chromosomes in *P. vitticeps*, as it is in snakes. Identification of the Z chromosome and investigation of its sequence composition will permit us to comment further on the evolutionary status of the sex chromosome system in *P. vitticeps*.

It is generally considered that highly differentiated sex chromosomes are a barrier to the subsequent evolution of TSD, and that homomorphic sex chromosomes are a necessary prerequisite for such a transition in a sex-determining mechanism (for explanation see Bull 1980, 1983, Janzen & Paukstis 1991). GSD is thought to progressively evolve from an initial state of sex chromosome homomorphy through increasing, and presumably irreversible, stages of differentiation, until a point of cytologically distinguishable sex chromosome heteromorphy is attained (Bull 1983, Charlesworth 1991). That is, once heteromorphic sex chromosomes have evolved, there is no returning to a state of homomorphism, and thus no possibility of an evolutionary switch to TSD.

However, the pattern of TSD and GSD amongst the Australian agamids indicates that transitions between the two modes have occurred frequently in their evolutionary history (Harlow 2001, Sarre *et al*. 2004). GSD species in such a group might therefore be expected to exhibit sex chromosomes at an early stage of differentiation, as presumably insufficient time will have elapsed following a switch from TSD to GSD for large-scale differences between the sex homologues to have accumulated. In this context, it is surprising to find that the W chromosome in *P. vitticeps* is highly differentiated from the Z, in terms of both heterochromatinization of the W and its apparent lack of sequence homology to the Z. Since the karyotype of *P. vitticeps* is representative of the Australian agamids, which have a highly conserved karyotype (Witten 1983), the evolutionary status of the sex chromosome system in this species is of particular relevance to questions of evolutionary transitions between the two modes of sex determination in this group of reptiles. If, as our findings suggest, the sex chromosomes in *P. vitticeps* are truly advanced, this may indicate that GSD reptiles with highly differentiated sex chromosomes can indeed switch to TSD, a phenomenon that would be in complete contradiction to the prevailing theory (Bull 1980, 1983, Janzen & Paukstis 1991). Our finding is further supported by the recent discovery of temperature-influenced sex ratios in the skink *Bassiana duperreyi* (Shine *et al*. 2002), a species with highly differentiated sex chromosomes (Donnellan 1985).

Our results suggest that microchromosomes, which are common in reptilian karyotypes, may hold the key to sex determination of many species whose sex chromosomes remain cryptic. Detailed investigation of microchromosomes might lead to the identification of cryptic sex chromosomes in many other GSD reptile species, whose karyotypes have already been described. We have already observed such a situation in an Australian turtle species, *Chelodina longicollis* (unpublished data). In many published studies, microchromosomes have been omitted from the karyotype because they are difficult to characterize, so their identification as sex chromosomes could have been overlooked. Therefore, very little information is available regarding their role in sex chromosomal evolution. Chromosome rearrangements involving microchromosomes may well play a major role in sex chromosome differentiation in the reptilian lineages. Further experiments are required to examine the sequences of such 'micro-sex-chromosomes'. Our isolation of DNA from the W chromosome of *P. vitticeps* by microdissection will allow us to develop a W-chromosome-specific library which may lead to the discovery of novel genes in the sex determination pathway of lizards and also in other related species. This may prove of immense benefit to our understanding of the evolution of sex chromosomes in vertebrates.

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