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ORIGINAL ARTICLE

Extension, single-locus conversion and physical mapping of sex chromosome sequences identify the Z microchromosome and pseudo-autosomal region in a dragon lizard, *Pogona vitticeps*

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Distribution of temperature-dependent sex determination (TSD) and genotypic sex determination (GSD) across the phylogeny of dragon lizards implies multiple independent origins of at least one, and probably both, modes of sex determination. Female *Pogona vitticeps* are the heterogametic sex, but ZZ individuals reverse to a female phenotype at high incubation temperatures. We used reiterated genome walking to extend Z and W chromosome-linked amplified fragment length polymorphism (AFLP) markers, and fluorescence *in situ* hybridization for physical mapping. One extended fragment hybridized to both W and Z microchromosomes, identifying the Z microchromosome for the first time, and a second hybridized to the centromere of all microchromosomes. W-linked sequences were converted to a single-locus PCR sexing assay. *P. vitticeps* sex chromosome sequences also shared homology with several other Australian dragons. Further physical mapping and isolation of sex-specific bacterial artificial chromosome clones will provide insight into the evolution of sex determination and sex chromosomes in GSD and TSD dragon lizards.
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Introduction

Sex in vertebrates is determined by genes on sex chromosomes (genotypic sex determination, GSD) and by environmental factors that exert influence during embryonic development (environmental sex determination), or a combination of both. GSD is ubiquitous in mammals and amphibians and is the most widespread form of sex determination in fish. The most common form of environmental sex determination in vertebrates is temperature-dependent sex determination (TSD) in which temperature during embryogenesis influences the sex of the offspring (Bull, 1983). Some fishes show TSD (Conover and Kynard, 1981; Devlin and Nagahama, 2002; Conover, 2004), but it is most prevalent in reptiles.

The reptiles are of particular interest because both TSD and GSD are widespread in this vertebrate group. TSD is shown by the tuatara (Cree *et al*., 1995), all crocodilians (Ferguson and Joanen, 1982; Deeming, 2004), many turtles (Bull and Vogt, 1979; Pieau, 1982; Ewert *et al*., 2004) and a minority of lizards (Charnier, 1966; Harlow, 2004). Female heterogametic GSD (ZZ males/ZW females) is apparently ubiquitous in snakes (Beçak *et al*., 1964; Ohno, 1967; Matsubara *et al*., 2006) whereas turtles and lizards with GSD have either female or male heterogamety (XY males/XX females), in some cases with multiple sex chromosomes (Solari, 1994). The heterogametic sex of many reptiles is yet to be determined because the sex chromosomes are homomorphic or cryptic (Ezaz *et al*., 2005, 2006b).

The diversity of sex-determining mechanisms and their almost haphazard distribution across the reptile phylogeny allude to a complex evolutionary history of transitions between male and female heterogametic GSD and between GSD and TSD (Bull, 1980; Kraak and Pen, 2002; Janzen and Krenz, 2004; Sarre *et al*., 2004; Ezaz *et al*., 2006a). This is true even within the family of dragon lizards (Agamidae), which are emerging as a model group for the study of sex determination evolution, sex ratio evolution and sex allocation (Harlow, 2004; Ezaz *et al*., 2005; Warner and Shine, 2005; Doody *et al*., 2006; Janzen and Phillips, 2006; Uller *et al*., 2006; Uller and Olsson, 2006; Quinn *et al*., 2007; Warner and Shine, 2007, 2008; Ezaz *et al*., 2009). They present an excellent opportunity to understand better the molecular and chromosomal basis of evolutionary transitions between the two fundamental mechanisms of vertebrate sex determination, TSD and GSD.

Reconstruction of such transitions requires a comparative approach in which the homology of sex chromosomes,

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sex genes and TSD mode can be contrasted and the direction and nature of transitions in sex determination modes inferred. A comparative analysis of sex-related sequences and chromosomes is perhaps the most tractable of these approaches, but it requires the initial identification of sex-related sequences. There are very few sex-specific sequences reported for reptiles (Cooper *et al*., 1997; Halverson and Spelman, 2002), although repetitive satellite sequences are known to be interspersed throughout the chromosomes of snakes in high copy number (Singh *et al*., 1976, 1980) and are concentrated in particularly high density on the W chromosome (Solari, 1994). A number of functional genes have recently been mapped to the Z and W chromosomes of three snakes, a gecko and a turtle (Matsubara *et al*., 2006; Kawaia *et al*., 2007; Kawagoshi *et al*., 2009).

Previously, we identified homologous Z and W chromosome amplified fragment length polymorphism (AFLP) markers for the Australian central bearded dragon lizard, *Pogona vitticeps* (Quinn *et al*., 2007). This species has a conserved karyotype that is typical of Australian agamids ($2n = 32$, comprising 12 macrochromosomes and 20 microchromosomes; Witten, 1983) and shows female heterogamety (Ezaz *et al*., 2005). In this paper, we have extended the *P. vitticeps* AFLP markers, Pv72W and Pv71Z (Quinn *et al*., 2007), into larger sex chromosome fragments using reiterated genome walking. Two of the reiterated genome-walked fragments were mapped to reveal their physical location in the genome. We converted the extended fragments into a high-throughput single-locus PCR sexing assay to enable analysis of the interaction between incubation temperature and genotype in the sex determination of *P. vitticeps*, and we tested for related sequences in other agamids.

Materials and methods

Animals and DNA extraction

The species, number of individuals, their phenotypic sex and collection sites are listed in Table 1. Phenotypic sex of hatchling *P. vitticeps* was determined using hemipene eversion (Harlow, 1996) and confirmed by dissection and examination of gonadal morphology. Adults of all other species were sexed on the basis of external morphology. All adult females used in this study were known to have been gravid. Animals were killed by intracranial injection of sodium pentobarbitone (Sigma-Aldrich, Castle Hill, NSW, Australia) before dissection for gonadal sexing (hatchlings) or collection of tissue for fibroblast culture (adults). Genomic DNA was extracted from whole blood (collected from the caudal vein), tail-tip tissues or liver tissues, using standard phenol–chloroform procedures (Sambrook and Russell, 2001).

Table 1 Species, collection sites and number of males and females used in this study

Species	*Collection site*	*Numbers of males and females used in this study*		*Total*
		Male	*Female*	
Pogona vitticeps	NSW	33	77	110
Pogona barbata	NSW	3	3	6
Pogona henrylawsoni	QLD	1	1	2
Pogona minor	WA	–	–	1
Amphibolurus muricatus	ACT, NSW	1	1	2
Amphibolurus nobbi	VIC	1	1	2
Ctenophorus pictus	NSW	1	1	2
Lophognathus longirostris	SA	1	1	2
Physignathus lesueurii	ACT	1	1	2

Abbreviations: NSW, New South Wales; QLD, Queensland; WA, Western Australia; ACT, Australian Capital Territory; VIC, Victoria; SA, South Australia.
Missing data = phenotypic sex unknown.

Isolation and genome walking of sex-linked amplified fragment length polymorphism (AFLP) fragments

Two sex-linked AFLP fragments (Pv72W and Pv71Z), identified previously (Quinn *et al*., 2007), were isolated and reamplified from a 5% high-resolution agarose gel using 'band-stab' PCR (Sambrook and Russell, 2001), and then isolated again by excision from a 1% low-melting-point agarose gel and subsequent digestion with β-agarase I enzyme (New England Biolabs, Ipswich, MA, USA). DNA was precipitated, purified, cloned and sequenced following standard protocols. Larger genomic fragments were generated from these AFLP fragments by a reiterated process of genome walking using reagents supplied in the GenomeWalker Universal Kit (Clontech Laboratories, Mountain View, CA, USA) as per the manufacturer's instructions. In brief, four genome walking 'libraries' were constructed by digestion of genomic DNA from one male and one female with a blunt-end restriction enzyme (*Dra*I, *Eco*RV, *Pvu*II or *Stu*I) and ligation of GenomeWalker adaptors to the restriction fragments. A set of 'gene-specific' primers (GSPs) was designed to anchor within the AFLP fragment sequences and to enable amplification in both directions (Supplementary Table 1). Each primary GSP, GSP1, was designed in combination with a nested secondary GSP, GSP2, on the same DNA strand. GSP1 primers were paired with adaptor primer 1 (BD Clontech) to amplify primary PCR products from genome walking libraries. GSP2 primers were paired with adaptor primer 2 (BD Clontech) to amplify secondary PCR products from the primary products. Products for seven secondary genome walking PCRs were excised from a 2% high-resolution agarose, cloned and sequenced. Nested primers were designed to anchor within the derived sequences and were used to continue sequencing through the genome walking fragments outwards into unknown flanking sequences. Sequences were assembled into contiguous fragments using Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA). Standard nucleotide blast and protein blast were used to search for homologous sequences. ExPASy protein analysis tools (Swiss Institute of Bioinformatics Quartier Sorge—Batiment Genopode 1015 Lausanne, Switzerland) were used for the translation and analysis of open reading frame.

Blood culture, chromosome preparation, fluorescence *in situ* hybridization (FISH) and C-banding

Short-term culture of whole or peripheral blood leukocytes, metaphase chromosome preparations, C-banding and FISH were performed as described by Ezaz *et al*.

Table 2 Comparisons between PCR sex assay (present study) and AFLP genotyping (Quinn *et al*., 2007) in *Pogona vitticeps*.

Origin	*Phenotype*	*Number of individuals*	*PCR genotype*		*AFLP genotype*	
			Male	*Female*	*Male*	*Female*
Wild-caught	Male	6	6	0	6	0
	Female	9	1	8	1	8
28 °C incubation	Male	25	24[a]	0	25	0
	Female	25	1	24	1	24
34°, 35° or 36° C incubation	Male	2	1[a]	0	2	0
	Female	43	20	23	20	23
Total		33 M, 77 F	53	55	55	55

Abbreviation: AFLP, amplified fragment length polymorphism.
[a]Unsuccessful PCR in one male.

(2005). The FISH probe was stripped from the slides following the protocol of Müller *et al*. (2002).

Single-locus conversion

A single-nucleotide polymorphism, distinguishing putative W and Z chromosome AFLP markers, was exploited to design a W chromosome-specific primer (primer F1, Supplementary Table 1). This was paired with a primer designed to anneal at a site in one of the genome walking contigs, common to males and females (primer J; Supplementary Table 1). A second pair of primers (Supplementary Table 1, primers E and C) was designed for the amplification of a larger fragment of another genome walking contig present in both sexes to serve as a positive control for amplification. The four primers were combined into a duplex PCR to identify chromosomal sex in *P. vitticeps* on the basis of amplification (or non-amplification) of the W chromosome fragment. PCR conditions were as follows: 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} each deoxyribonucleotide triphosphate, 5 pmol of each primer, 0.5 U of BioTaq Red DNA polymerase (Bioline, Alexandria, New South Wales, Australia) and 2 μl of the accompanying 10 × PCR buffer were added to 20–50 ng of genomic DNA template in a reaction volume of 20 μl, and cycled in a touchdown PCR (94 °C for 2 min, then 10 cycles of 94 °C for 30 s, 67 °C for 30 s decreasing by 0.5 °C per cycle, and 72 °C for 1 min, then 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 5 min). These PCR conditions were used in subsequent PCRs unless specified otherwise. The duplex PCR was tested on 110 *P. vitticeps* for which putative chromosomal sex had previously been determined using AFLP genotyping ($n = 77$ ZW; 33 ZZ; 9 W chromosomes from unrelated individuals; Table 2; Quinn *et al*., 2007).

Cross-species amplification of *P. vitticeps* single-locus sex marker

The PCR assay designed to identify chromosomal sex in *P. vitticeps* was also tested on eight related agamid species, including three other *Pogona* species (Table 1). As a further test for the presence of homologous sequences in other agamids, PCRs that amplified nested fragments of one of the genome walking contigs were tested on five agamid species (one of each sex) outside the *Pogona* genus (Supplementary Table 1; primer pairs A1–L1 and F–H2). PCRs were as described above, except that the cycling conditions were 94 °C for 2 min, then 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 2 min, followed by 72 °C for 5 min.

Results

Fragment extension, sequence assembly and analyses

Sequences from 12 genomic fragments generated by genome walking were assembled into five contigs. Two contigs were eliminated from further analyses because they were generated by an incorrect (mispaired) secondary primer sequences (data not shown), leaving three contigs potentially representing sex chromosome sequences (contigs A, B and C, comprising secondary products 1–5, Figure 1). We determined that these three contigs were not overlapping or adjacent (that is, A-B, A-C or B-C; data not shown) by comparing sequences and undertaking PCR experiments with nested primers.

Analyses of contig C sequences revealed an 18-bp indel between two fragments generated from the female genome walking libraries (fragments 4 and 5; Figure 1). PCR amplification from all males and females using primers specific to fragment 4 (Primer pair J–F1; Supplementary Table 1) revealed that fragment to contain sequences from the W chromosome only. Fragment 5 was thus presumed to be homologous Z chromosome sequences (Figure 2). Primer J was not only found to be female-specific amplifying fragment 4 from females only ($n = 10$ males; 10 females incubated at 28 °C), but was also found to be present in two of six wild-caught males (data not shown). Additional sequencing from fragment 4 extended this contig to 4.5 kb (fragments 7–12; Figure 2), and identified further high homology between the Z and W chromosome sequences.

PCR experiments using a Pv72W-specific primer (Supplementary Figure 1 and Supplementary Table 1) indicated that contig A, similar to contig C, represents homologous Z and W chromosomal sequences (Figure 2).

Sequence analysis of genome walking contigs

Nucleotide and protein BLAST analyses of contig A (2.2 kb; Genbank accession EU938136), and contig C (4.5 kb: 3.288 kb-C1 + 1.094 kb C1; Genbank accessions EU938138/EU938139) revealed no sections of significant similarity to database sequences, with the exception of a 185-bp section of contig C that showed a 68% nucleotide similarity to sections of several bacterial artificial chromosome library clones containing chicken (*Gallus gallus*) Z chromosome sequences. Repeat masking (http://www.girinst.org/cgi-bin/censor) revealed this section to be highly similar to the chicken repeat 1 (CR1) repetitive element. We obtained the sequences for

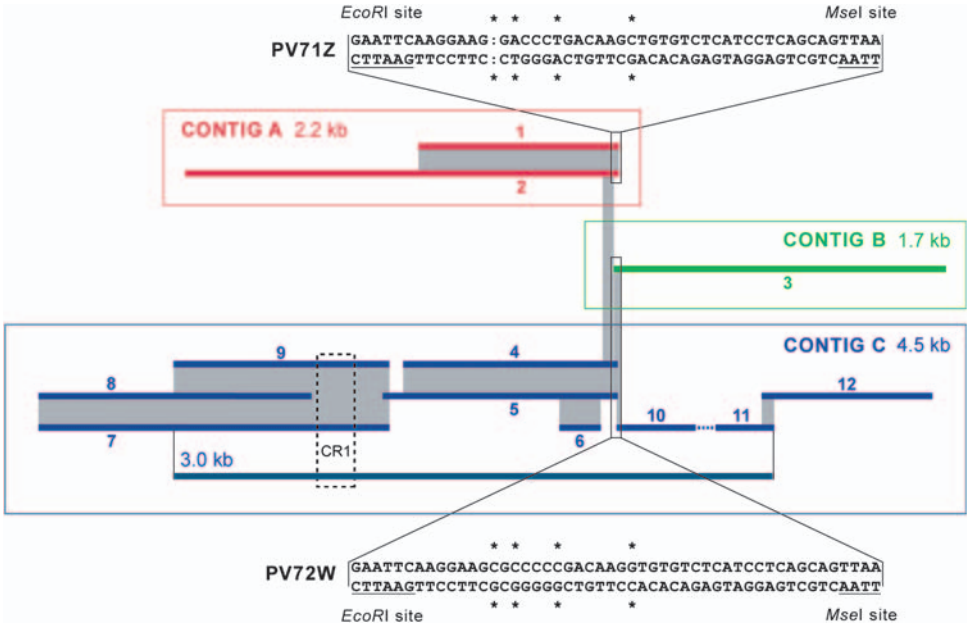

Figure 1 Genome walking products (shown to scale) generated from male and female *Pogona vitticeps* assembled into three distinct contigs: A (red), B (green) and C (blue). Vertical black boxes indicate sites of Pv71Z and Pv72W amplified fragment length polymorphism (AFLP) sequences. Bars 1–5 are cloned and sequenced fragments generated in a first phase of genome walking and bars 6–12 are sequences obtained in a second phase of genome walking to extend contig C. Grey shading shows areas of perfect or very high sequence homology; thus, these contigs share no homology outside the AFLP sequences except for a short section of contigs A and C just beyond the *Eco*RI site. A 3-kb PCR amplicon of contig C used in fluorescence *in situ* hybridization (FISH) experiments is indicated. The dotted line between fragments 10 and 11 indicates the section that was not sequenced because of C–G repeats. Dashed line box indicates chicken repeat 1 (CR1)-like repetitive element. * Indel and single-nucleotide polymorphism (SNP) differences between the two AFLP sequences.

this repetitive element for three female and two male *P. vitticeps*. In both males and one female, the CR1-like element was 188 bp in length, but an identical 14-bp deletion reduced the length of the repeat element at this locus to 174 bp in the other two females.

Contigs A and C were analyzed for protein similarity. Nucleotide sequences were translated (http://au.expasy.org/tools/dna.html) and the largest open reading frames (A: 242 aa, C: 120 aa; data not shown) from contigs A and C were used to identify homology for protein sequences (http://au.expasy.org/tools/blast/?VIRT28858; Blastp). No significant amino acid similarity was identified.

Fluorescence *in situ* hybridization (FISH)

Contig C (3-kb fragment) hybridized to one pair of microchromosomes (Figures 3b and c), including the heterochromatic W identified by subsequent C-banding (two males, two females; Figure 3d). The other member of the pair was thus revealed through homology to be the Z. Contig A produced specific hybridization signals to the centromere of all microchromosomes (Figure 3e), as well as non-specific signals to all macrochromosomes. This indicates the presence of microchromosome centromere-specific common repeats in addition to other repeats common to all macrochromosomes.

Single-locus conversion and sex assay

The single-locus PCR sex assay was applied to a total of 110 *P. vitticeps*, all of which were previously tested using AFLP genotyping (Quinn *et al*., 2007). Primer F1 was designed to be W specific—the 3′ terminal nucleotide aligns with one of the single nucleotides distinguishing the Pv72W and Pv71Z AFLP sequences (Figure 2). In the PCR sex assay, primer F1 was paired with primer F4 (Supplementary Table 1) to amplify a 224-bp fragment diagnostic of the W chromosome, and primers C and E (Supplementary Table 1) were paired to amplify a 963-bp fragment of contig B (Figure 2) as a positive control. Amplification of the 224-bp female-specific product was expected to be favored over the 963-bp positive control product because of its smaller size. Scoring of the PCR sex assay results on agarose gels resulted in complete agreement between AFLP and PCR genotypes (Table 2).

Cross-species PCR analyses

The PCR sexing assay developed for *P. vitticeps* identified sex correctly for *Pogona barbata* and *P. henrylawsoni* (Table 1; Figure 4a). The positive control product also amplified in the single individual of *P. minor* (of unknown sex) available for analysis. Thus, it is plausible that the PCR sexing assay developed for *P. vitticeps* is applicable to all seven species of *Pogona*. The control band amplified (with the size expected for *P. vitticeps*) in *Amphibolurus muricatus* and *A. nobbi*, but not in the other agamid species from outside the *Pogona* genus. In *A. nobbi*, a faint band of the size expected for the W chromosome product for *P. vitticeps* amplified for the one female examined, suggesting that there may be significant homology between the sex chromosomes of *Pogona* and *A. nobbi* (Figure 4a).

PCR amplification with primers A1–L1 (Supplementary Table 1) yielded products of varying sizes for the five species outside the *Pogona* genus, but as for

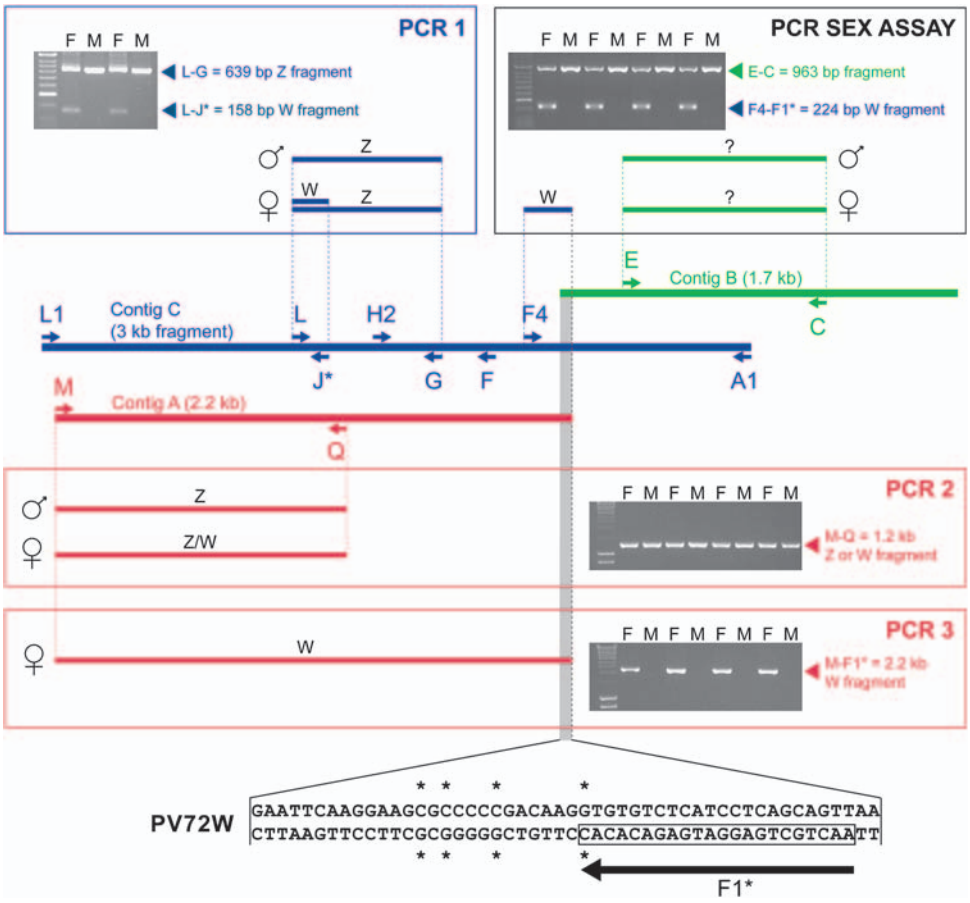

Figure 2 Genomic PCR products (and agarose gels) for male (M) and female (F) *Pogona vitticeps* and location of the primers derived from contigs A (green) and B (red), and 3-kb fragment amplified from contig C (blue; see Figure 2). Letters denote primers (Supplementary Table 1) with arrows indicating direction of primer extension (5′-3′). The three contig sequences do not overlap with the exception of the shared Pv72W/Pv71Z amplified fragment length polymorphism (AFLP) sequences (grey shading). PCR 1 determined that contig C represented homologous Z/W sequences, as primer J was female-specific (*) for an entire clutch. The boxed sequences indicates the female-specific (*) F1 primer (within the Pv72W sequences) that was used to determine that contig A represents homologous Z/W sequences (PCR 2 and PCR 3) and to amplify a W chromosome-specific band in the PCR sex assay. The PCR sex assay also amplified a fragment of contig B as a positive control. PCR experiments to verify contig B as sex chromosome sequences were unsuccessful (data not shown).

P. vitticeps, they amplified a 3-kb product in *P. barbata* females and most males but showed intraspecific variation by amplifying a considerably larger fragment in one male (Figure 4b). In contrast, PCR amplification with primers F–H2, which amplify a 522-bp fragment of contig C in *P. vitticeps*, amplified quite similar-sized products for all the agamid species tested with the exception of *Lophognathus longirostris*, and the male *A. nobbi* (Figure 4c).

Discussion

Agamid lizards hold considerable promise as a model group for deciphering the processes and genomic changes underpinning the evolution of vertebrate sex-determining mechanisms and sex chromosomes. Comparative analysis of agamid sex chromosomes and identification of their homologues in TSD agamids requires the development of comparative genomic tools, including hybridization probes for conserved sex chromosome sequences.

We successfully used genome walking to generate larger genomic sequences (up to 4.5 kb) from 71-bp and 72-bp sex-linked AFLP markers in *P. vitticeps*. We also developed a robust PCR test for high-throughput sex genotyping. The PCR sex assay for *P. vitticeps* is the first such test developed for agamids and only the second W chromosome-specific marker reported for reptiles (Halverson and Spelman, 2002).

We identified the Z chromosome in *P. vitticeps* for the first time. We also confirmed our previous proposition that the Z is a microchromosome, based on the lack of unpaired chromosomes visible at meiosis and no evidence for heteromorphism in the macrochromosomes (Ezaz *et al*., 2005). Our data suggest that, for the most part, the W and Z chromosomes of *P. vitticeps* are highly differentiated, with the microdissected W chromosome probe hybridizing poorly to the Z chromosome and a high degree of heterochromatinization evident in the W (Ezaz *et al*., 2005).

Although the Z and W chromosomes in *P. vitticeps* are clearly differentiated, our data also show that the Z and W have retained some homologous sequences that are

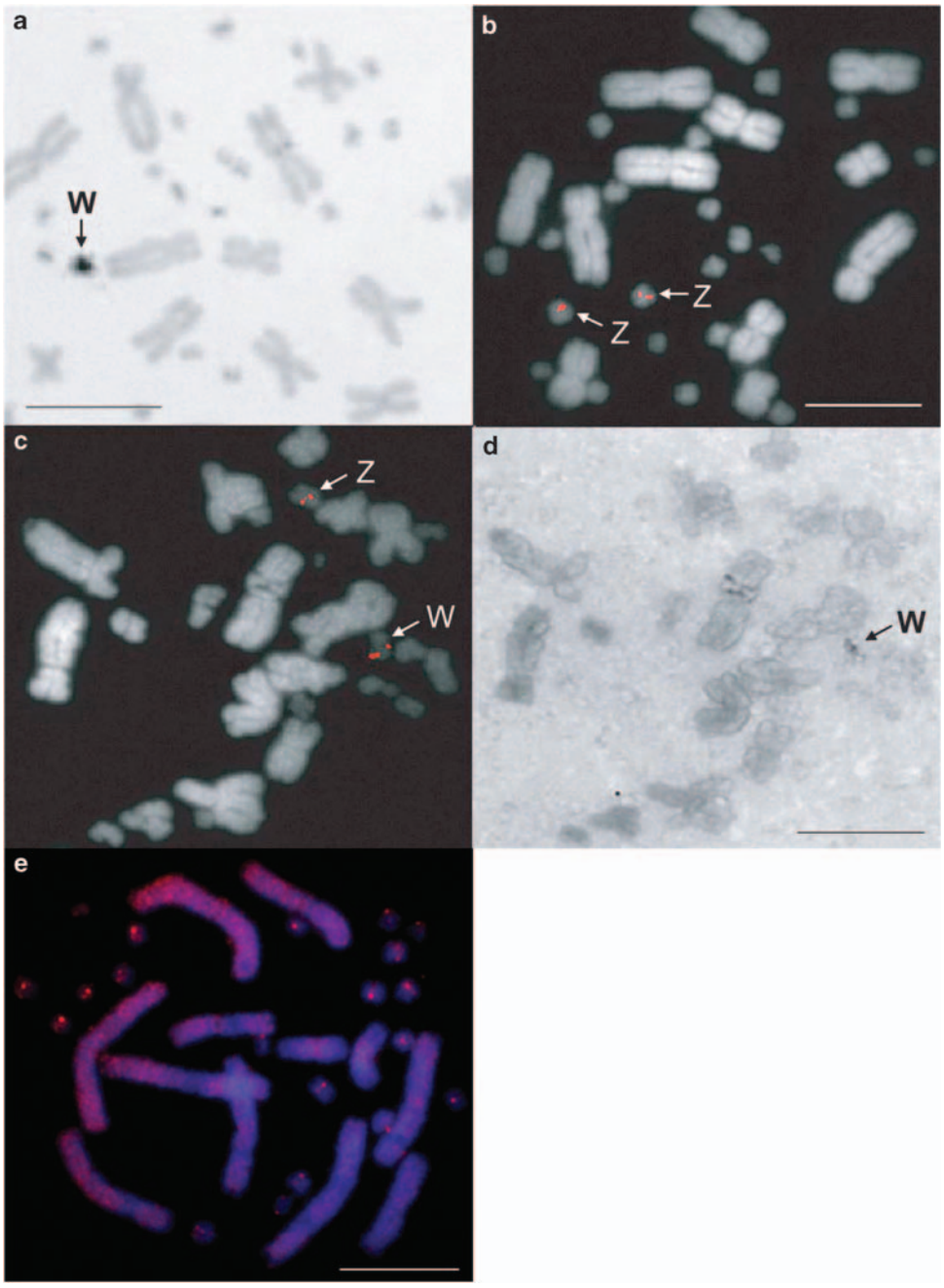

Figure 3 C-banding and physical mapping of contigs A and C onto metaphase chromosome spreads of *P. vitticeps*. (**a**) C-banded female identifying the W chromosome. Contig C mapped onto Z microchromosome pair in male (**b**) and Z and W microchromosomes in female (**c**). (**d**) C-banding of same female metaphase spread identifying only the W chromosome. (**e**): Physical mapping of contig A onto female metaphase showing centromere-specific hybridization in microchromosomes and nonspecific hybridization in macrochromosomes. Scale bar represents 10 μm.

very closely related. A number of explanations exist for the maintenance of this sequence homology but it is likely that contig C forms part of, or was recently closely linked to, a pseudo-autosomal region in this species.

With the notable exception of the CR1-like repetitive element, we found no evidence for coding sequences in the *P. vitticeps* sex chromosome sequences. The CR1-like repetitive element belongs to the non-long terminal repeat class of retrotransposons. CR1-like elements seem to be ubiquitous in vertebrates (Vandergon and Reitman, 1994; Kajikawa *et al*., 1997; Poulter *et al*., 1999; Jurka, 2000) and they are also present in invertebrates (Drew and Brindley, 1997). It is possible that the amplified 3-kb probe, which contained the sequences for the 188-bp CR1-like element, hybridized preferentially to CR1-like elements on the Z and W chromosomes. If so, that would imply that this repetitive element is concentrated in high copy number on the sex chromosomes of *P. vitticeps* relative to the autosomes. Further FISH experiments, with a sex chromosome probe lacking this repetitive element, are required to address this question.

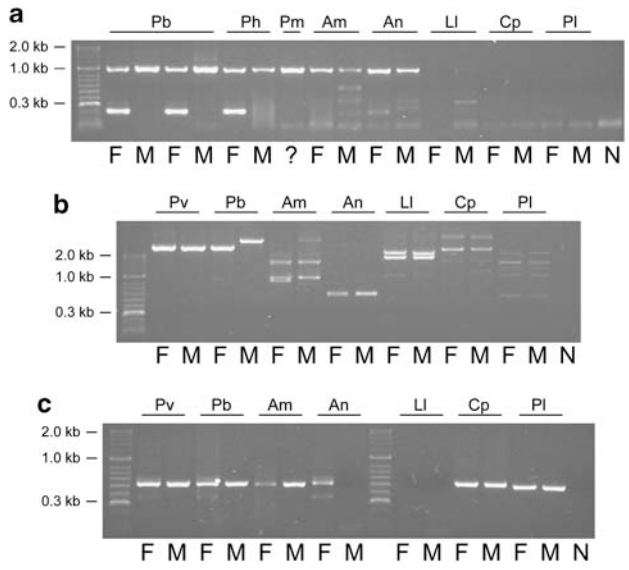

Figure 4 Agarose gels showing PCR tests in Australian agamids using primers from sex chromosome sequences isolated from *P. vitticeps*. Panel a shows *P. vitticeps* PCR sexing assay tested on eight other species. Panel b shows amplification using the primers for the 3-kb fragment of contig C (A1–L1; Supplementary Table 1) in five other agamid species. Panel c shows amplification using primers for a nested 522-bp fragment of contig C (F–H2; Supplementary Table 1). Am, *Amphibolurus muricatus*; An, *A. nobbi*; Cp, *Ctenophorus pictus*; F, female; Ll, *Lophognathus longirostris*; M, male; N, negative control; Pb, *P. barbata*; Ph, *P. henrylawsoni*; Pl, *Physignathus lesueurii*; Pm, *P. minor*; Pv, *Pogona vitticeps*. Molecular weight markers are shown on the left.

The microchromosomal centromeric location of contig A in *P. vitticeps* suggests that its origin is related to a mutation or chromosomal rearrangement (for example, inversion) near the centromere. They are probably lizard-specific repeats because no homology to annotated repeats in existing databases (http://www.repeatmasker.org/) could be found. Specific hybridization to the sex chromosomes could not be shown, presumably because signals of the repeats on the other chromosomes obscured the hybridization signals of any unique sex chromosome-specific sequences. Therefore, contig A may contain unique sex chromosome sequences as well as centromeric repeats common to all microchromosomes. Further analysis, including sequencing of already identified bacterial artificial chromosome clones (Ezaz *et al*., unpublished data) as well as cross-species FISH mapping using contig A as a probe, is required to fully characterize these sequences.

We found that contigs A and C are apparently distinct chromosomal fragments and not in juxtaposition, even though each contains a Pv72W sequence. This suggests that Pv72W is an amplified or repetitive locus on the W chromosome. The fluorescence intensity of the Pv72W marker was conspicuously higher than for all other products in the AFLP profile (Quinn *et al*., 2007), consistent with this hypothesis. If Pv72W is indeed multilocus, this would complicate inferences about linkage between this locus and the non-recombining region of the W chromosome. Further work will clearly be necessary to fully characterize the Pv72W locus. Southern hybridization to male and female genomic DNA, and additional FISH experiments (with subsequent C-banding), using amplified fragments of the three contigs as probes, may shed light on their chromosomal location, and confirm if the Pv72W marker is part of amplified sequences on the W chromosome.

Our PCR experiments amplifying homologous sequences in other agamid species strongly suggest that other Australian agamids may have sequences homologous to the sex chromosome sequences of *P. vitticeps*. Indeed, we recently performed comparative FISH experiments using the amplified 3-kb fragment of contig C as a hybridization probe to deduce sex chromosome homology among 12 species of Australian agamids (Ezaz *et al*., 2009). This identified independent origins of ZZ/ZW sex chromosome systems among GSD dragons and identified chromosomes in TSD dragons that are homologous to the sex chromosomes of *P. vitticeps*. Thus, the molecular tools described in this paper have already enabled a first step toward reconstructing the genomic changes that have occurred in evolutionary transitions between GSD and TSD in the Agamidae. Further studies to analyze the homology of these *P. vitticeps* sex chromosome sequences in more distantly related taxa might help to reveal the origins of sex chromosomes and sex-related genes in reptiles.

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