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The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds

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Abstract Reptiles show a diverse array of sex chromosomal systems but, remarkably, the Z sex chromosomes of chicken are homologous to the ZW sex chromosomes of a species of gecko, *Gekko hokouensis*, suggesting an ancient but common origin. This is in contrast to the ZW sex chromosomes of snakes and a species of soft-shelled turtle, *Pelodiscus sinensis*, which are nonhomologous to those of chicken or each other and appear to have been independently derived. In this paper, we determine what homology, if any, the sex chromosomes of the Australian dragon lizard *Pogona vitticeps* shares with those of snake and chicken by mapping the dragon homologs of five snake Z chromosome genes (*WAC*, *KLF6*, *TAX1BP1*, *RAB5A*, and *CTNNB1*) and five chicken Z chromosome genes (*ATP5A1*, *GHR*, *DMRT1*, *CHD1*, and *APTX*) to chromosomes in the dragon. The dragon homologs of snake and chicken sex chromosome genes map to chromosomes 6 and chromosome 2, respectively, in the dragon and that *DMRT1*, the bird sex-determining gene, is not located on the sex chromosomes of *P. vitticeps*. Indeed, our data show that the dragon homolog to the chicken Z chromosome is likely to be wholly contained within chromosome 2 in *P. vitticeps*, which suggests that the sex-determining factor in *P. vitticeps* is not the sex-determining gene of chicken. Homology between chicken Z chromosome and *G. hokouensis* ZW chromosome pairs has been interpreted as retention of ancient ZW sex chromosomes in which case the nonhomologous sex chromosomes of snake and dragons would be independently derived. Our data add another case of independently derived sex chromosomes in a squamate reptile, which makes retention of ancient sex chromosome homology in the squamates less plausible. Alternatively, the conservation between the bird Z chromosome and the *G. hokouensis* ZW chromosomes pairs is coincidental, may be an example of convergent evolution, its status as the Z chromosome having been independently derived in birds and *G. hokouensis*.

Keywords agamidae · comparative gene mapping · dmrt1 · FISH · sex determination · BAC

Abbreviations

BAC	bacterial artificial chromosomes
DAPI	4′,6-diamidino-2-phenylindole

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dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytidine 5′-triphosphate
dGTP	2′-deoxyguanosine 5′-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dUTP	2′-deoxyuridine 5′-triphosphate
FISH	fluorescent in situ hybridization
GSD	genotypic sex determination
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Overgo	overlapping oligonucleotides
PCR	polymerase chain reaction
SSC	standard saline citrate
TSD	temperature-dependent sex determination
v/*v*	volume/volume

Introduction

Reptiles show a remarkable diversity in sex chromosomes and sex determination. In some reptiles, as well as some fish, sex is determined by the temperature experienced by the developing embryos (temperature-dependent sex determination [TSD]). In many others, it is determined by the genetic complement the individual receives at conception (genotypic sex determination [GSD]). In others, genotype and environment can interact to determine sex (Quinn et al. 2007; Radder et al. 2008). GSD reptiles are either male heterogametic (i.e., XX females and XY males) as in humans or female heterogametic (ZZ males and ZW females) as in birds and snakes. The almost haphazard distribution of these modes of sex determination, including TSD, across the reptile phylogeny (Ezaz et al. 2009; Organ and Janes 2008; Pokorná and Kratochvíl 2009) have led to suggestions that evolutionary transitions between these modes are relatively easily achieved (Barske and Capel 2008; Ezaz et al. 2006; Georges et al. 2009; Sarre et al. 2004) and that each mode may have evolved independently many times (Ezaz et al. 2009; Organ and Janes 2008; Pokorná and Kratochvíl 2009). TSD may, in some cases, be an intermediary state that facilitates the subsequent capture of sex determination by novel sex chromosomes from autosomes (Georges et al. 2009) and be a significant contributor to the diversity thus far seen in the sex chromosomes of reptiles.

The comparative genomic approach has provided important insights into the evolution of sex chromosome and sex determination in vertebrates (Chowdhary et al. 1998; Kawagoshi et al. 2009; Kawai et al. 2007; Matsubara et al. 2006; Matsuda et al. 2005; Nanda and Schmid 2002; Shetty et al. 1999; Veyrunes et al. 2008). For example, comparative gene mapping has determined the homology of chicken sex chromosomes largely to human chromosomes 9 and 5 with small regions of 18, 4, and 8 (Nanda et al. 2002; Schmid et al. 2005) and established an independent origin for the chromosomes carrying sex-determining genes in birds, snakes, a turtle, and mammals (Kawai et al. 2007; Matsubara et al. 2006; Matsuda et al. 2005).

The bird Z chromosome is extremely well conserved among different bird families (Graves and Shetty 2001; Ohno et al. 1964; Shetty et al. 1999; Stiglec et al. 2007), and similarly, the Z chromosome is well conserved among snake families (Becak and Becak 1969; Becak et al. 1964; Singh 1972). Initially, it was thought that bird and snake Z chromosomes were homologous to snake Z chromosomes (Ohno 1967). However, comparative gene mapping has recently shown the bird Z chromosome to be homologous to chromosome 2 in the rat snake, *Elaphe quadrivirgata*; conversely, the rat snake Z chromosome is homologous to chromosomes 2 and 27 in chicken (Matsubara et al. 2006). Similarly, the chicken Z chromosome has homology to chromosome 6 in the Chinese soft-shelled turtle (*Pelodiscus sinensis*) rather than to the sex microchromosomes (Kawai et al. 2007), which is found to be homologous to chicken chromosome 15 (Kawagoshi et al. 2009). Clearly, the conservation in large syntenic regions across vertebrate chromosomes does not translate to the conservation of the sex-determining function of a particular chromosome.

Remarkably, however, the sex chromosomes of chicken and a species of Japanese gecko *Gekko hokouensis* have recently been found to share a syntenic region containing six genes in conserved order, suggesting a highly conserved homology in sex chromosomes between birds and lizards (Kawai et al. 2009). Even more remarkably, the XY sex chromosome complex of monotreme mammals shares homology with the bird Z chromosome (Veyrunes et al. 2008), suggesting it was present in the common ancestor of mammals and birds 300 million years ago (Graves 2008). Thus, there is evidence from comparative gene mapping for retention of ancient plesiomorphic states among the sex chromosomes of mammals, birds, and reptiles, as well as for multiple and independently derived states (Ezaz et al.

2009; Kawagoshi et al. 2009; Kawai et al. 2009, 2007; Matsubara et al. 2006; Matsuda et al. 2005).

The evolutionary lability of the sex chromosomes and mechanisms of sex determination in reptiles (Ezaz et al. 2006; Organ and Janes 2008; Pokorná and Kratochvíl 2009; Sarre et al. 2004) is at odds with the apparent retention, over many hundreds of millions of years, of ancestral sex chromosomes and perhaps mechanism of sex determination in monotreme mammals, *G. hokouensis*, and birds. The alternate explanation that must be considered is that the genes and mechanisms of sex determination are independently derived, but coincidentally come to reside on the chromosomes homologous with chicken sex chromosomes. Without comparing the role of *DMRT1* and associated mechanisms involved in sex determination in chicken to that of *G. hokouensis* and platypus, we cannot tell whether the same gene is responsible for sex determination or whether different genes on the same chromosome have been recruited to this function.

Comparative mapping in other lizards can help distinguish the conservation of sex chromosomes through descent from convergent evolution. Homology of the sex chromosomes of lizards, whether ZZ/ZW or XX/XY (Quinn et al., submitted), to the bird Z chromosome would favor the hypothesis of a deep ancestral origin, perhaps in common with birds, platypus, and even eutherian mammals. Conversely, nonhomologous sex chromosome in other lizard families would favor convergence in sex chromosomes between birds and *G. hokouensis.*

In this study, we apply comparative candidate gene mapping approaches to determine cross-species homology between the sex chromosomes of chicken and snake and the recently identified sex chromosomes of the Australian central bearded dragon *Pogona vitticeps. P. vitticeps* has a ZZ/ZW sex microchromosome system with a highly heterochromatic W microchromosome and euchromatic Z microchromosome (Ezaz et al. 2005; Quinn et al. 2009). For this study, we selected ten genes based on their distribution along the sex chromosomes in snakes and chicken (Matsubara et al. 2006; http://www.ensembl.org/index.html). Snake genes were selected to represent the entire short arm of the snake Z chromosome, whereas chicken genes were selected to represent the entire Z chromosomes. We screened a *P. vitticeps* genomic BAC library to isolate homologs of genes from bird and snake sex chromosomes and physically mapped these BAC clones onto *P. vitticeps* chromosomes by fluorescence in situ hybridization (FISH). We report that sex chromosomes in *P. vitticeps* are not homologous to the Z chromosomes of chicken, snakes, or *G. hokouensis*, suggesting independently derived sex chromosomes in this dragon lizard species.

Materials and methods

Cell culture, chromosome preparation, genes, and overgos

The relevant protocols for sexing *P. vitticeps*, cell culture, and chromosome preparation have been described elsewhere (Ezaz et al. 2008, 2005). *P. vitticeps* BAC clones containing homologs of snake and chicken sex chromosome genes were isolated using overlapping oligonucleotides (overgo)-based genomic library screening (Ross et al. 1999). Genes mapped in this study, their locations in the genomes of snake and chicken, and overgo sequences are presented in Table 1. *Anolis carolinensis* genome sequences were used as a reference to design overgos for screening a female *P. vitticeps* genomic BAC library with 6.2 times coverage (commissioned via commercial vendor Amplicon Express; http://www.genomex.com/). Conserved sequences for each gene were obtained by multiple alignment using Ensembl Genome Browser (http://www.ensembl.org/index.html). The consensus-conserved sequence was then compared against the *A. carolinensis* genome using Blat (http://genome.ucsc.edu/) to identify homologous sequences. Prospective 40-bp overgo sequences were then designed using OvergoMaker (http://bioinf.wehi.edu.au/cgi-bin/overgomaker) and their quality assessed (e.g., presence of repetitive sequences, homology with random short sequences by analyzing the *E* value) using blast against *A. carolinensis* genome assembly (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Overgo labeling, *P. vitticeps* BAC library screening, dot blots, and identification of positive clones

Overgos were labeled with α^{32}P dATP and α^{32}P dCTP. Briefly, 5 µl annealing mix was prepared containing 20 µM of both the forward and reverse overgo that were denatured at 80°C for 5 min and then annealed at 37°C for 10 min. The annealed overgos were then

Table 1 Physical location and overgo sequences for snake and chicken Z chromosome genes including the number of BAC clones isolated and mapped in *P. vitticeps*

Gene	Chromosomal location		Overgo sequences	Number of BAC clones isolated in *P. vitticeps*	
	Snakes[a]	Chicken		Isolated	Mapped
WAC	Zp	2p	Overgo a: GGAAGAGAAGCATTCCAGTGATGC Overgo b: TGGGAGCATATTAGCGGCATCACT	2	2
KLF6	Zp	2p	Overgo a: AGTCAGGAGGATCTGTGGACCAAA Overgo b: CGCGTGCCAGAATAATTTTGGTCC	8	8
TAX1BP1	Zp	2p	Overgo a: CAATGGAAGATGAAGGAAATTCTG Overgo b: GTCACCACCAACATATCAGAATTT	4	4
RAB5A	Zp	2p	Overgo a: GGGAGAATCTGCAGTTGGAAAGTC Overgo b: ACGAAGCACCAGACTTGACTTTCC	2	2
CTNNB1	Zp	2p	Overgo a: ATGGACTTCCAGTGGTGGTCAAAC Overgo b: GATGGTGGATGCAAGAGTTTGACC	3	3
ATP5A1	2p	Zp	Overgo a: CTCTGCTGAGCTAGAAGAAACTGG Overgo b: AATTGAAAGCACGCGGCCAGTTTC	2	2
GHR	2p	Zp	Overgo a: CAGTTCAAACCCAGCTAAGCAACC Overgo b: TTTGCCACTGAACTCTGGTTGCTT	7	4
DMRT1	2p	Zp	Overgo a: AAAGGGCACAAGCGGTTCTGCATG Overgo b: ACTGGCAATCTCTCCACATGCAGA	3	3
CHD1	2p	Zq	Overgo a: GCAGGCTATCCAGATTATTACTGC Overgo b: GAAGACCCTGCCATTTGCAGTAAT	1	1
APTX	–	Zq	Overgo a: CACCTCCATCTTCATGTTATCAGC Overgo b: CAGAATCAAAATCCTGGCTGATAA	1	1
Total				33	30

Gene orders in both snake and chicken are from short arm to long arm

[a] Location and gene order in snake are based on that of Z chromosomal location in the Japanese rat snake *E. quadrivirgata* (Matsubara et al. 2006; Kawai et al. 2007)

placed on ice and 10 μl labeling mix was prepared by adding 0.5 μl (2 mg/ml) BSA, 2 μl OLB solution (1 part solution A: 1 ml solution O, 18 ml β-mercaptoethanol, 5 ml 100 mM dNTP, 5 ml 100 mM dGTP; 2.5 parts solution B: 2 M HEPES–NaOH, pH 6.6; 1.5 parts solution C: 3 mM Tris–HCL, pH 7.4, 0.2 mM ethylenediaminetetraacetic acid [EDTA]), 0.5 μl (50 μCi) each ^{32}P dATP and ^{32}P dCTP, and 0.5 μl Klenow fragment (2.5 U). This mix was added to the annealed overgos to a volume of 10 μl (4.5 μl annealed overgos+5.5 μl labeling mix) and incubated at room temperature for at least 1 h. Unincorporated nucleotides were removed by passing through Sephadex columns (GE Healthcare) following the manufacturer's instructions. The labeled probe was denatured for 10 min at 95°C and placed on ice for at least for 2 min before adding to 25 ml hybridization buffer (1 mM EDTA, 7% sodium dodecyl sulfate [SDS], and 0.5 M phosphate buffer).

High-density *P. vitticeps* BAC filters were prehybridized at 60°C with hybridization buffer for at least 1 h before labeled probe was added. The prehybridization buffer was discarded and 25 ml fresh hybridization buffer containing label probe mix was added. Up to 20 probes were used for single hybridizations on up to four filters inside a standard hybridization bottle. Hybridizations were performed at 60°C for at least 18 h.

The filters were washed four times at 60°C with 100 ml wash solutions as follows: 1×30 min in wash solution B (1 mM EDTA, 1% SDS, and 40 mM Na_2HPO_4); 2×20 min in was solution 2 (1.5× standard saline citrate [SSC], 0.1% SDS), 1×20 min wash solution 3 (0.5× SSC, 0.1% SDS). After the last wash, filters were removed from the bottle, rinsed in 2× SSC, pat dried, wrapped in cling film, and placed inside an autoradiography cassette with intensifying screen and X-ray film. The cassettes were stored at

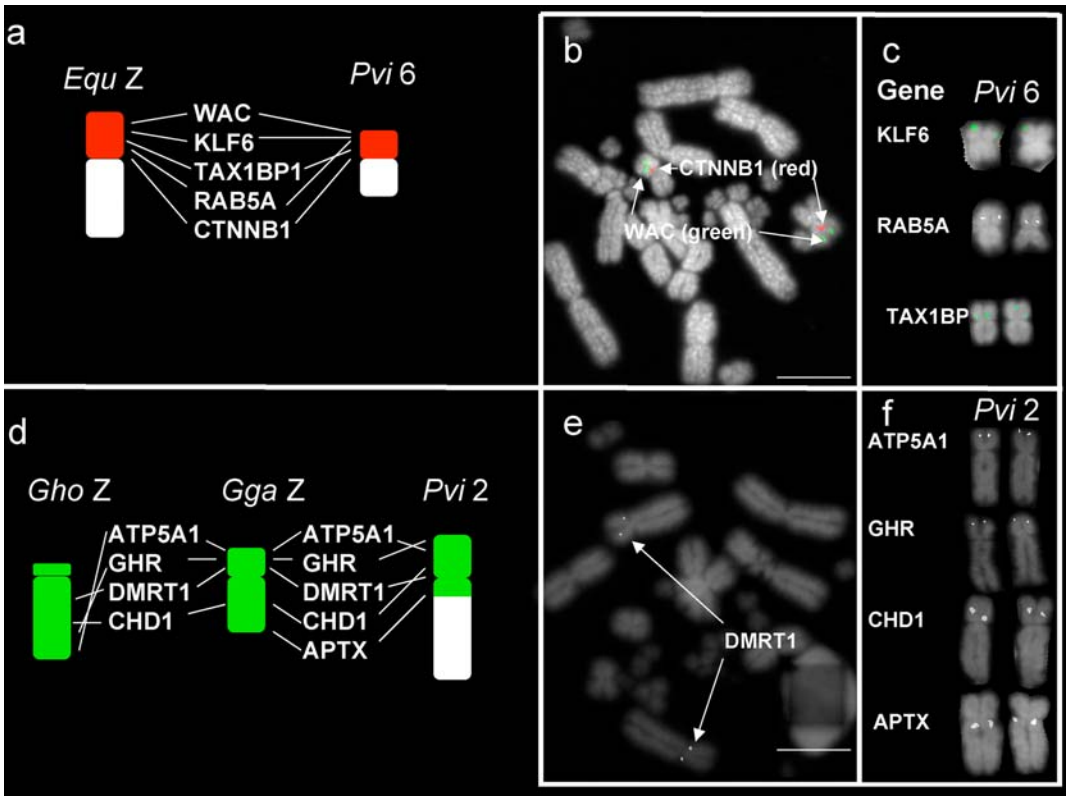

Fig. 1 Physical mapping of *P. vitticeps* homologs of five snake and chicken Z chromosome genes in the Australian dragon lizard *P. vitticeps*. **a** Schematic diagram showing the locations of *P. vitticeps* homologs of five snake Z chromosome genes onto the short arm (p arm) of autosome 6 in *P. vitticeps*. **b** Two-color FISH showing the hybridizations of BAC clones containing *CTTNB1* (*red*) and *WAC* (*green*) onto chromosome 6p in *P. vitticeps*. **c** *Top* to *bottom* hybridizations of BAC clones containing the genes *KLF6*, *RAB5A*, and *TAX1BP1* onto chromosome 6p in *P. vitticeps*. **d** Schematic diagram showing the locations of *P. vitticeps* homologs of five chicken Z chromosome genes onto autosome 2 in *P. vitticeps*. **e** *P. vitticeps* metaphase chromosome spread showing hybridization of BAC clone containing the *DMRT1* gene onto chromosome 2p. **f** *Top* to *bottom* chromosome 2 in *P. vitticeps* showing the hybridizations of BAC clones containing the genes *ATP5A1*, *GHR*, and *CHD1* onto the short arm and *APTX* onto the proximal part of long arm of chromosome 2. *Equ E. quadrivirgata*, *Pvi P. vitticeps*, *Gho G. hokouensis*, *Gga Gallus gallus*. Scale bar represent 10 μm

−80°C and films were developed after 1 day to 2 weeks following standard procedure. The coordinates of positive BAC clones were identified according to the manufacturer's instructions.

BAC DNA extraction, labeling, and gene mapping by FISH

Positive BAC clones were streaked on Luria–Bertani (LB) agar–chloramphenicol plate and grown overnight at 37°C. A single colony was grown overnight in 15 ml liquid culture (LB broth) with appropriate amount of chloramphenicol. The BAC DNA was extracted using the Qiagen miniprep kit following the manufacturer's instructions. Approximately, 250–500 ng BAC DNA was labeled using nick translation. The protocols for nick translation, FISH, image capture, and analysis were performed as described by Ezaz et al. (2009).

Results

We screened a *P. vitticeps* genomic BAC library to isolate *P. vitticeps* homologs of five genes from snake sex chromosomes (*WAC*, *KLF6*, *TAX1BP1*, *RAB5A*, and *CTNNB1*) and five genes from chicken sex chromosomes (*ATP5A1*, *GHR*, *DMRT1*, *CHD1*, and *APTX*; Matsubara et al. 2006; http://www.ensembl.org/index.html). We physically mapped these BAC

clones containing *P. vitticeps* homologs of snake and chicken sex chromosome genes by FISH onto the metaphase chromosomes in *P. vitticeps*.

Isolation of BAC clones containing *P. vitticeps* homologs of snake and chicken sex chromosome genes

A total of 33 BAC clones containing *P. vitticeps* homologs of ten sex chromosome genes from snake and chicken were isolated. The number of BAC clones isolated for each gene in *P. vitticeps* ranged from one to eight (Table 1). Individual *P. vitticeps* BAC clones containing orthologous genes from snake sex and chicken Z chromosomes were identified by dot blots using the same overgo pair used for BAC library screening. For *Dmrt1*, an additional PCR was also performed with the primers designed against conserved intronic regions A, B, and C (data not shown). Where there was more than one positive BAC clone for a single gene, at least two BACs were physically mapped to confirm their chromosomal locations (data not shown). In all cases, multiple BAC clones identified for a single gene mapped to the same region of the same chromosomes, revealing that they represent overlapping BACs. Our approach to use the fully sequenced *A. carolinensis* as a reference yielded moderate success (40–60%; data not shown) in identifying BAC clones containing genes of interest in *P. vitticeps*.

Chromosome locations of *P. vitticeps* homologs of snake sex chromosomal genes

BAC clones containing *P. vitticeps* homologs of five snake sex chromosome genes (*WAC*, *KLF6*, *TAX1BP1*, *RAB5A*, and *CTNNB1*) were physically mapped by FISH in *P. vitticeps* (Fig. 1a–c). These clones all mapped onto the short arm of chromosome 6. A minimum of 30 metaphase spreads were analyzed for each BAC clone and hybridization signal was found to be consistent in all cells analyzed. A two-color FISH with BAC clones containing genes *WAC* and *CTNNB1* was also performed to reveal the corresponding location of these two genes onto chromosome 6 in *P. vitticeps*. The gene order of these five genes onto *P. vitticeps* chromosome 6 (*WAC–KLF6–RAB5A–TAX1BP1–CTNNB1*) is very similar to that found on the Z chromosome of the Japanese rat snake *E. quadrivirgata* (*WAC–KLF6–TAX1BP1–RAB5A–CTNNB1*) with the exception of a rearrangement between *RAB5A* and *TAX1BP1* (Fig. 1a–c). However, these genes were found to be highly rearranged when compared to Z chromosomes of the Burmese python *Python molurus* and the pit viper *Trimeresurus flavoviridis* (see Matsubara et al. 2006).

Chromosome locations of *P. vitticeps* homologs of chicken Z chromosomal genes

BAC clones containing *P. vitticeps* homologs of five chicken Z chromosome genes (*ATP5A1*, *GHR*, *DMRT1*, *CHD1*, and *APTX*) were physically mapped by FISH in *P. vitticeps* (Fig. 1d–f). All five genes mapped onto chromosome 2. A minimum of 30 metaphase spreads were analyzed for each BAC clone and hybridization signal was found to be consistent in all cells analyzed. The gene order of these five genes onto *P. vitticeps* chromosome 2 was found to be different (*GHR–ATP5A1–CHD–DMRT1–APTX*) to that of chicken Z chromosome (*ATP5A1–GHR–DMRT1–CHD1–APTX*; Fig. 1d–f). Three of the genes from the short arm of chicken Z chromosome, *ATP5A1*, *GHR*, and *DMRT1*, were hybridized onto the short arm of

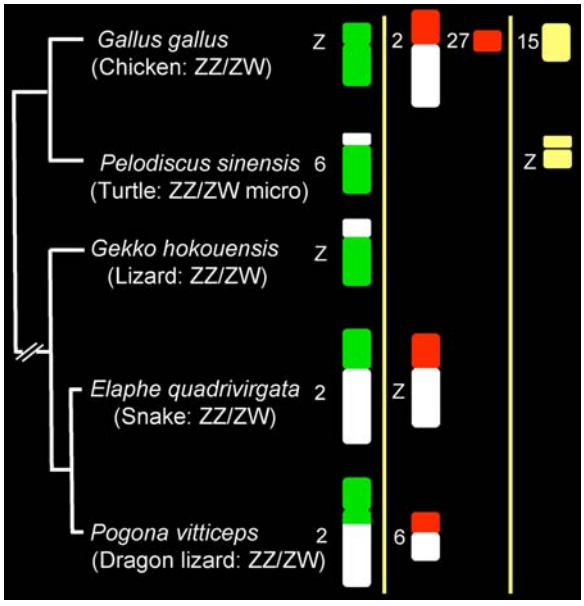

Fig. 2 Schematic representation showing the nonhomology of ZW sex chromosomes in reptiles based on reciprocal mapping of sex chromosomal genes from chicken, snakes, and turtle. Data are summarized after Matsuda et al. (2005), Matsubara et al. (2006), Kawai et al. (2007, 2009), Kawagoshi et al. (2009), the current study, and http://www.ensembl.org/index.html

chromosome 2 in *P. vitticeps*, whereas two genes, *CHD1* and *APTX*, from the long arm of the chicken Z chromosome mapped onto the distal and proximal region of chromosome 2 (Fig. 1d–f).

Thus, sex chromosomes in *P. vitticeps* are not homologous to the Z chromosomes of chicken (or gecko, *G. hokouensis*) or snakes, suggesting independently derived sex chromosomes in this second lizard species.

Discussion

Mechanisms of sex determination in Australian agamid lizards are highly diverse. There seem to have been numerous transitions between GSD and TSD modes, as well as within GSD modes, since at least two types of ZW sex chromosomes have been identified among related taxa (Ezaz et al. 2009). By applying comparative genomic approaches, we have shown here that the ZW sex microchromosomes found in *P. vitticeps* (the most common type of Z chromosome observed among agamids) share no homology with the Z chromosomes of chickens, *G. hokouensis*, or snakes.

Rather, our data identify the short arm of the snake Z chromosome to be homologous to the short arm of chromosome 6 in *P. vitticeps* and the Z chromosomes in chickens and *G. hokouensis* to be homologous to chromosome 2 in *P. vitticeps* (Fig. 1). Although *P. vitticeps* chromosome 2 is homologous to the chicken Z, gene order is different from that conserved between chicken and *G. hokouensis*, implying that gene rearrangements have occurred between the chicken/*G. hokouensis* and *P. vitticeps* lineages (Figs. 1a–c and 2). Since *ATP5A1* and *APTX* lie at opposite distal points of the chicken Z chromosome and *APTX* is located on the proximal part of the long arm of chromosome 2 (near the centromere) in *P. vitticeps* (Fig. 1d, f), thus it is likely that the entire chicken Z chromosome is contained entirely within chromosome 2. It is, therefore, unlikely that a region of the ancestral Z fused with an autosome to create a neo-ZW in agamid lizards.

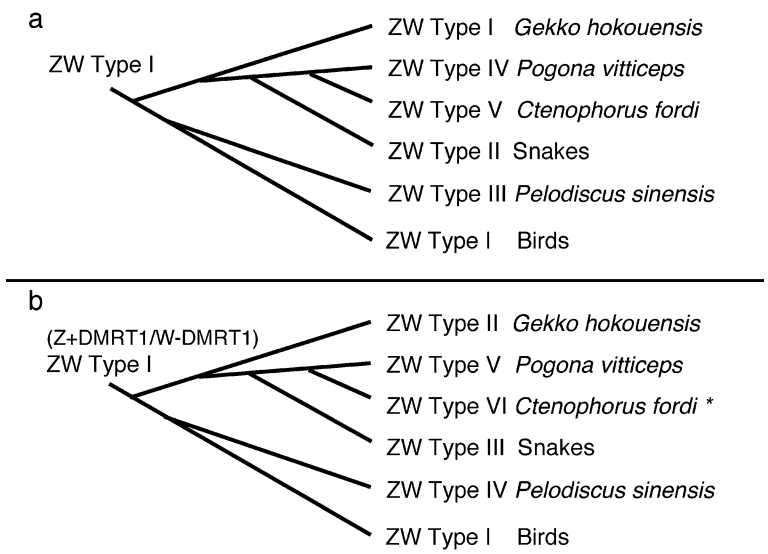

Fig. 3 Evidence of multiple and independent origins of ZZ/ZW sex chromosome systems in reptiles based on gross sex chromosome homology as revealed by gene mapping and the presence or absence of *DMRT1* (male-determining gene in chicken) on the sex chromosomes. **a** Putative evidence of five independent origins of ZW sex chromosomes (*types I–V*) in reptiles (data summarized from the combined reciprocal gene mapping data from selected snake and chicken sex chromosomal genes and mapping data from sex chromosome DNA probe derived from *P. vitticeps*); **b** putative evidence of six independent origins of novel ZW sex chromosomes in reptiles (*types I–VI*) by capturing novel sex-determining genes (as revealed by the presence or absence of the *DMRT1* gene—the male-determining gene in birds). Note: *DMRT1* is present only on Z chromosomes in birds, on both ZW chromosomes in *G. hokouensis*, and autosomal in snakes (chromosome 2), turtle (*P. sinensis*; chromosome 6), and dragon lizard (*P. vitticeps*; chromosome 2). Data are summarized from Matsuda et al. (2005), Matsubara et al. (2006), Kawai et al. (2007, 2009), Ezaz et al. (2009), Smith et al. (2009), the current study, and http://www.ensembl.org/index.html. The *asterisk* denotes predicted—mapping data unavailable

Our comparative mapping implies that the agamid lizard *P. vitticeps* does not share sex chromosomes or sex-determining genes with birds, *G. hokouensis*, or snakes (Fig. 3a). The presence of *DMRT1*, the male-determining gene in birds (Smith et al. 2009), on the sex chromosomes of *G. hokouensis* suggests a commonality with birds in sex determination itself, but its presence on both the Z and W chromosomes makes a role as the master sex-determining factor unlikely (Fig. 3b). The identification, through our analysis, of an autosomal location for *DMRT1* in *P. vitticeps* makes a sex-determination role for this gene even less likely (Fig. 3b). Indeed, *DMRT1* is likely to be important in the sex-determining pathway in *P. vitticeps*, as it is in all other vertebrates (and maybe in invertebrates), but its absence from the sex chromosomes makes it an unlikely candidate for the master sex-determining gene.

If the birds and *G. hokouensis* Z chromosomes are truly conserved, then the non-homology of snakes and *P. vitticeps* Z chromosomes suggest that the sex-determination function have been assumed by novel genes. Alternatively, the conservation between the bird and the *G. hokouensis* Z chromosomes may be an example of convergent evolution, its status as the Z chromosome having been independently derived in birds and *G. hokouensis*. This possibility becomes more likely given the non-homology in Z chromosomes among *P. vitticeps*, snakes, birds, and *P. sinensis* (Kawagoshi et al. 2009; Kawai et al. 2009, 2007; Matsubara et al. 2006; Matsuda et al. 2005). This lack of homology has occurred either through the coincidental recruitment of sex determination by one or more genes on this chromosome or the translocation of key sex-determining elements to the autosome that then became the Z chromosome in *G. hokouensis*. Either way, the autosomal location of snake Z genes in *P. vitticeps* shows that sex determination involved different genes on different chromosome regions, so were likely to have been independent events in these taxa (Figs. 1a–c and 3b).

The similarities in size and morphology between the snake Z chromosome and a combined chromosome 6 and the Z microchromosome in *P. vitticeps* (Ezaz et al. 2009; Quinn et al. 2009) makes it plausible that the Z microchromosome of *P. vitticeps* originated from an ancestral Z chromosome common to snakes and lizards, which took their current conformation through chromosome rearrangements (fission or fusion). If that is indeed the case, then it is likely that snakes and *P. vitticeps* share the same sex-determining genes. Reciprocal cross-species gene mapping of more snake Z chromosomal genes and genes from the *P. vitticeps* ZW microchromosomes could resolve that issue.

These findings, along with an earlier observation of the existence of at least two types of ZW chromosomes in agamids, support the proposition that the evolution of sex determination in agamids is both rapid and recent (Ezaz et al. 2009). Our results do not support the proposition that birds and lizards share a deep ancestral homology in sex chromosomes but instead provide evidence of multiple and independent evolution of sex chromosomes in squamates (Fig. 3). Additional mapping of more genes from snake and chicken sex chromosomes among various ZW (e.g., other geckos, lacertids, and varanids) and XY (e.g., geckos, skinks, iguanids, and pygopods) lizards will reveal the homology and evolution of sex chromosomes in squamate reptiles and resolve the competing interpretations for the syntenic homology of chicken and *G. hokouensis* ZW chromosomes—ancient chromosomal homology and mechanism of sex determination or ancient chromosomal homology with more recent independent acquisition of sex-determining function via the recruitment of novel sex-determining genes and chromosomes.

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