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Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards

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Abstract Distribution of sex-determining mechanisms across Australian agamids shows no clear phylogenetic segregation, suggesting multiple transitions between temperature-dependent (TSD) and genotypic sex determination (GSD). These taxa thus present an excellent opportunity for studying the evolution of sex chromosomes, and evolutionary transitions between TSD and GSD. Here we report the hybridization of a 3 kb genomic sequence (PvZW3) that marks the Z and W microchromosomes of the Australian central bearded dragon (*Pogona vitticeps*) to chromosomes of 12 species of Australian agamids from eight genera using fluorescence in-situ hybridization (FISH). The probe hybridized to a single microchromosome pair in 11 of these species, but to the tip of the long arm of chromosome pair 2 in the twelfth (*Physignathus lesueurii*), indicating a micro-macro chromosome rearrangement. Three TSD species shared the marked microchromosome, implying that it is a conserved autosome in related species that determine sex by temperature. C-banding identified the marked microchromosome as the heterochromatic W chromosome in two of the three GSD species. However, in *Ctenophorus fordi*, the probe hybridized to a different microchromosome from that shown by C-banding to be the heterochromatic W, suggesting an independent origin for the ZW chromosome pair in that species. Given the haphazard distribution of GSD and TSD in this group and the existence of at least two sets of sex microchromosomes in GSD species, we conclude that sex-determining mechanisms in this family have evolved independently, multiple times in a short evolutionary period.

Keywords GSD · TSD · reptile · sex microchromosomes · evolution · FISH · C-banding

Abbreviations

DAPI	4′,6-diamidino-2-phenylindole
dUTP	2′-deoxyuridine 5′-triphosphate
ESD	environmental sex determination
FISH	fluorescence in-situ hybridization
GSD	genotypic sex determination
PCR	polymerase chain reaction
SSC	standard saline citrate
TSD	temperature sex determination
v/v	volume/volume

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Introduction

Sex can be determined by genetic factors (genotypic sex determination, GSD), environmental factors (environmental sex determination, ESD) (Charnier 1966; Pieau 1971; Bull 1983), and in some cases, by an interaction between genotype and environment (Conover and Kynard 1981; Quinn et al. 2007; Radder et al. 2008). In many vertebrates, a primary sex-determining gene on a specific chromosome pair (the sex chromosomes) provides the initial trigger for sex determination (Sinclair et al. 1990; Matsuda et al. 2002), whereas in other vertebrates, sex determination depends on an environmental variable experienced during embryonic development, such as temperature, pH or salinity (Charnier 1966; Pieau 1971; Devlin and Nagahama 2002).

GSD vertebrates typically have either a male heterogametic (XY male/XX female) or a female heterogametic (ZZ male/ZW female) sex chromosomal system. The sex homologues (X and Y, or Z and W) may be highly differentiated, or differ only in a restricted region (even a single locus), as is expected given their autosomal origin. The XX/XY sex chromosome pair is conserved in therian mammals, and a ZZ/ZW pair is conserved in birds. In contrast, many reptile, amphibian and fish lineages exhibit remarkable variation in the sex chromosome pair, and in the system of heterogamety, sometimes even among closely related species or even populations (for review see Ezaz et al. 2006; Graves 2006). Most turtles, a minority of lizards, all crocodilians and the tuatara exhibit temperature-dependent sex determination (TSD), in which incubation temperature during egg development determines sex. GSD appears to be exhibited by all snakes, most lizards and a minority of turtles (Modi and Crews 2005; Ezaz et al. 2006), involving either male or female heterogamety (Solari 1994). Some lizards and snakes display more complex male or female heterogametic systems involving multiple sex chromosomes in varying evolutionary stages of differentiation.

Comparative mapping of sex chromosomal genes or sequences across phylogenetically distinct taxa by fluorescence in-situ hybridization can provide valuable information on the origin and evolution of sex chromosomes and sex-determining mechanisms. Recently, orthologues of chicken Z genes were mapped to autosomes in three species of snakes and in the soft-shelled turtle *Pelodiscus sinensis*, indicating that the ZW sex chromosomes of birds are not homologous with the ZW macrochromosome pair common to all snakes or with the ZW microchromosomes of this turtle (Matsubara et al. 2006; Kawai et al. 2007). However, Kawai et al. (2008) showed that in a ZW population of the gecko *Gekko hokouensis*, the Z chromosome shares six genes with the chicken Z, raising the possibility of a bird-like ZW system in an ancient reptile. It appears that different ancestral autosomes gave rise to the sex chromosomes of snakes and of *Pelodiscus sinensis* from the pair that became the bird and *G. hokouensis* ZW.

The only reports of lizard sex chromosome sequences include the six genes mapped in a gecko by Kawai et al. (2008), an X-linked microsatellite in Australian skinks (Cooper et al. 1997; Stow et al. 2001), a W chromosome sequence from the Asian varanid *Varanus komodoensis* (Halverson and Spelman 2002), a Y chromosome sequence in the Australian skink *Bassiana duperreyi* (unpublished observations) and sequences common to the Z and W microchromosomes of the Australian bearded dragon lizard, *Pogona vitticeps* (Agamidae) (Quinn et al. 2007). Substantial sex chromosome sequence data have already started to emerge from the genome sequencing project for the green anole lizard, *Anolis carolinensis* (http://www.broad.mit.edu/models/anole/).

Two groups (Janzen and Krenz 2004; Organ and Janes 2008) have recently reconstructed the evolutionary history of TSD and GSD within the Reptilia by mapping the occurrence of these mechanisms onto the phylogeny. Both groups considered GSD to be the most parsimonious ancestral condition for squamate reptiles (lizards and snakes). However, this conclusion should be treated cautiously because GSD and TSD may be omnibus states that (a) obscure diversity in underlying mechanisms and so fail to separate convergence from homology; (b) are labile and therefore subject to frequent reversals that render parsimony a blunt instrument; and (c) include some questionable classifications of TSD versus GSD (see Harlow 2004). In the absence of robust data on sex-determining mechanisms from sufficient representative taxa, a complementary approach to determining ancestry of sex-determining mechanisms is comparative mapping of sex chromosome sequences over a

shorter evolutionary timescale within appropriate reptilian lineages.

Dragon lizards (family Agamidae) include both GSD and TSD taxa (Charnier 1966; Ganesh and Raman 1995; Harlow and Shine 1999; Harlow 2000, 2004; Harlow and Taylor 2000; El Mouden et al. 2001; Uller and Olsson 2006; Uller et al. 2006). Australian agamids (ca. 70 species; Cogger 2000) represent a recent radiation (~25 Mya) from an Asian ancestor (Hugall et al. 2008), and show a distribution of GSD and TSD mechanisms suggesting an evolutionary history involving multiple independent origins of one, and possibly both, of these mechanisms of sex determination. Both GSD and TSD species can occur within the same genus (Harlow 2000, 2004; Harlow and Taylor 2000; Uller and Olsson 2006, Uller et al. 2006), and in at least one species, the central bearded dragon (*Pogona vitticeps*), there is an interaction between genotype and egg incubation temperature in sex determination (Quinn et al. 2007).

Karyotypes are highly conserved among Australian agamids, with most species having 6 macrochromosome and 10 microchromosome pairs (Witten 1983). Female heterogamety has been established for *Pogona vitticeps.* A highly heterochromatic W microchromosome was identified by comparative genomic hybridization and C-banding (Ezaz et al. 2005). Previously, we reported the isolation and physical mapping by FISH of a novel 3 kb sequence (PvZW3; GenBank accession EU938136) common to the Z and W microchromosomes of *Pogona vitticeps* (Quinn et al. submitted). Sequence database search using BLAST did not reveal any significant similarity, but repeat masker identified a 185 bp chicken CR1-like repeat element.

In the present study, we identified ZZ/ZW sex microchromosome systems in three other GSD dragon species by C-banding. Physical mapping of PvZW3 in these species, followed by C-banding of the same slides, indicates that two other GSD species share the ZW chromosomes of *P. vitticeps*, but a different sex microchromosome pair occurs in another species. We also physically mapped PvZW3 in another six GSD species with cryptic sex chromosomes and for three TSD species. Our findings suggest multiple origins of TSD and GSD, as well as independent evolution of sex chromosomes in Australian agamids.

Materials and methods

Animals, sexing, cell culture, chromosome preparations

A total of 12 agamid species representing 8 genera were collected from various locations around Australia (Table 1). Six of these species have GSD, three have TSD, and in the remaining three species the sex-determining mechanism is uncertain. One male and one female were examined from each of the species except for *Diporiphora bilineata* and *Tympanocryptis pinguicolla* (one male only) and *Chlamydosaurus kingii* (one female only).

Animals were euthanized by intraperitoneal injection of sodium pentobarbitone at a concentration of 150 μg/g body weight. Phenotypic sex was determined on the basis of external morphology, hemipene eversion (Harlow 1996), and by internal examination of gonadal morphology. Fibroblastic cultures were established from macerated explants of eye, pericardium or tail tip tissue. For larger animals, 0.2–1 ml whole blood was collected by caudal venepuncture and subjected to short-term lymphocyte culture. Cell culture and chromosome preparations were performed as described in Ezaz et al. (2005).

C-banding

To identify heterochromatic sex chromosomes, C-banding was examined in nine of the 12 species. Three species were not subjected to C-banding because only one sex was available (Table 1). The procedure was performed following the protocol described in Ezaz et al. 2005.

Probe preparation and fluorescence in-situ hybridization (FISH) followed by C-banding

The novel 3 kb sex chromosome-borne probe, PvZW3, derived from *Pogona vitticeps* was amplified from female genomic DNA by PCR as described in Quinn et al. (submitted). The PCR product was purified using a QIAquick kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Purified probe was labelled by incorporating SpectrumRed-labelled dUTP (Abbott Molecular, Abbott Park, IL, USA) by nick translation and

Table 1 List of species examined and their modes of sex determination

Species	Collection area[a]	Sex determination	Experiments				References for SD
			C-banding	FISH	FISH/C-banding	Number of animals used (m+f)	
Amphibolurus nobbi	Vic	GSD, ZZ/ZW	•	•	•	2+2	Harlow (2001), Current study
Diporiphora bilineata	NT	GSD		•		1+0	Harlow (2001)
Pogona barbata	NSW	GSD, ZZ/ZW	•	•	•	2+2	Harlow (2001), Current study
Tympanocryptis pinguicolla	ACT	Unknown		•		1+0	
Chlamydosaurus kingii	NT	TSD		•		0+1	Harlow and Shine (1999), Harlow (2001)
Amphibolurus norrisii	Vic	GSD	•	•		2+3	Harlow (2001)
Amphibolurus muricatus	ACT	TSD	•	•		2+3	Harlow (2001)
Lophognathus longirostris	SA	Unknown	•	•		1+1	
Ctenophorus fordi	NSW	GSD, ZZ/ZW	•	•	•	3+3	Harlow (2000), Uller and Olsson (2006), Current study
Ctenophorus pictus	NSW	GSD	•	•		3+3	Harlow (2000), Uller et al (2006)
Ctenophorus nuchalis	NSW	Unknown	•	•		2+2	
Physignathus lesueurii	ACT	TSD	•	•		2+2	Harlow (2001)

[a] ACT, Australian Capital Territory; NSW, New South Wales; NT, Northern Territory; SA, South Australia; Vic, Victoria.

precipitated as described in Ezaz et al. 2005. The probe was resuspended in hybridization buffer (1× Denhardt's solution, 50% v/v deionized formamide, 10% v/v dextran sulfate, 2× SSC, 40 mM sodium phosphate buffer pH 7.0), denatured, and hybridized onto denatured metaphase chromosomes overnight at 37°C. The slides were washed once in 0.4× SSC/0.3% IGEPAL (CA630) (Sigma-Aldrich, St Louis, MO, USA) at 60°C for 2–3 min, then once in 2× SSC/0.1% IGEPAL at room temperature for 1–2 min. Slides were dehydrated through an ethanol series (1 min each in each of a 70%, 90% and 100% solution), air dried, stained with DAPI (50 μg/ml DAPI solution in 2× SSC) for 30–45 s at room temperature) and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Vernier co-ordinates of each metaphase were recorded and images of 3–10 cells were captured. At least 20–30 cells were analysed microscopically for each individual. Slide analysis and imaging were performed as described in Ezaz et al. 2005.

To test whether PvZW3 hybridizes to the sex chromosomes of other GSD species, we performed FISH followed by C-banding on the same slide (Quinn et al. submitted) for one female of the three species *Pogona barbata*, *Amphibolurus nobbi* and *Ctenophorus fordi*, in which heteromorphic W chromosomes can be identified by C-banding. Briefly, the coverslips from the slides subjected to FISH were removed by soaking in 2× SSC followed by two 5 min washes in 2× SSC at room temperature. Slides were then dehydrated through an ethanol series (3 min in each of a 70%, 90% and 100% solution), air dried and subjected to C-banding as described in Ezaz et al. 2005. Slides were analysed under bright-field microscopy to reveal the concordance or discordance of PvZW3 probe localization and C-banded W chromosomes.

Results

The chromosomes of nine species of Australian agamid lizards were subjected to C-banding to identify heterochromatic sex chromosomes. Twelve species were probed with the sex-chromosomal fragment isolated from *Pogona vitticeps* (Table 1).

C banding

C-banding identified highly heteromorphic micro-chromosomes in females of *Pogona barbata*, *Amphibolurus nobbi* and *Ctenophorus fordi* as well as *Pogona vitticeps*. Each of these species had a

heterochromatic microchromosome in females that was absent from the chromosome complement of males, and so was identified as a W chromosome. This establishes a ZZ/ZW microchromosomal system in these species, similar to that found for *Pogona vitticeps* (Fig. 1). As for *Pogona vitticpes*, the Z chromosome has no heterochromatin that can be identified by C-banding. The other six species that were investigated in this study showed no sex-specific C-bands (data not shown).

Hybridization of *Pogona vitticeps* sex chromosomal probe

The sex chromosomal probe PvZW3 hybridized to a single pair of microchromosomes in 11 species studied. The sole exception was *Physignathus lesueurii*, in which a bright and dispersed hybridization signal was observed on the distal end of the long arm of chromosome 2 in both sexes (Fig. 2).

To determine whether this probe identified the sex chromosomes in other species, we performed sequential FISH and C-banding in females of *Pogona barbata*, *A. nobbi* and *Ctenophorus fordi* (Fig. 2). In *Pogona barbata* and *A. nobbi*, the FISH probe marked a single microchromosome pair, one member of which was identified as the W by C-banding (the other being Z). However, the hybridization of PvZW3 in *Ctenophorus fordi* was to a microchromosome pair other than the C-banded W, indicating an autosomal location for the probe. As C-banding identifies only the heterochromatic W, we cannot identify the Z in this species (Fig. 2).

Discussion

We examined C-banding in nine Australian agamid species in an attempt to identify sex chromosomes and examine their homology. For six of these species

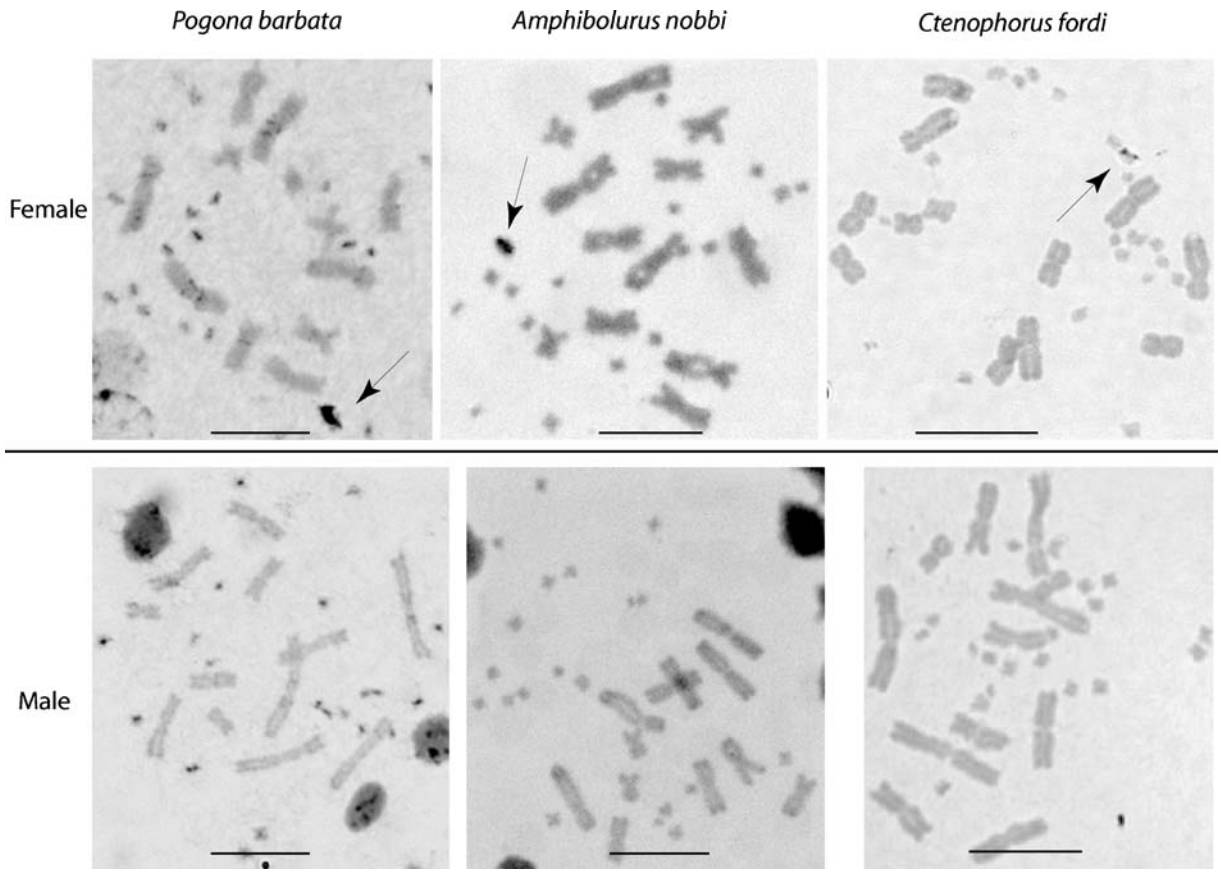

Fig. 1 C-banded metaphase chromosomes in three species of Australian dragon lizards. Arrows indicate heterochromatic W chromosomes in females. Scale bar represents 10 μm

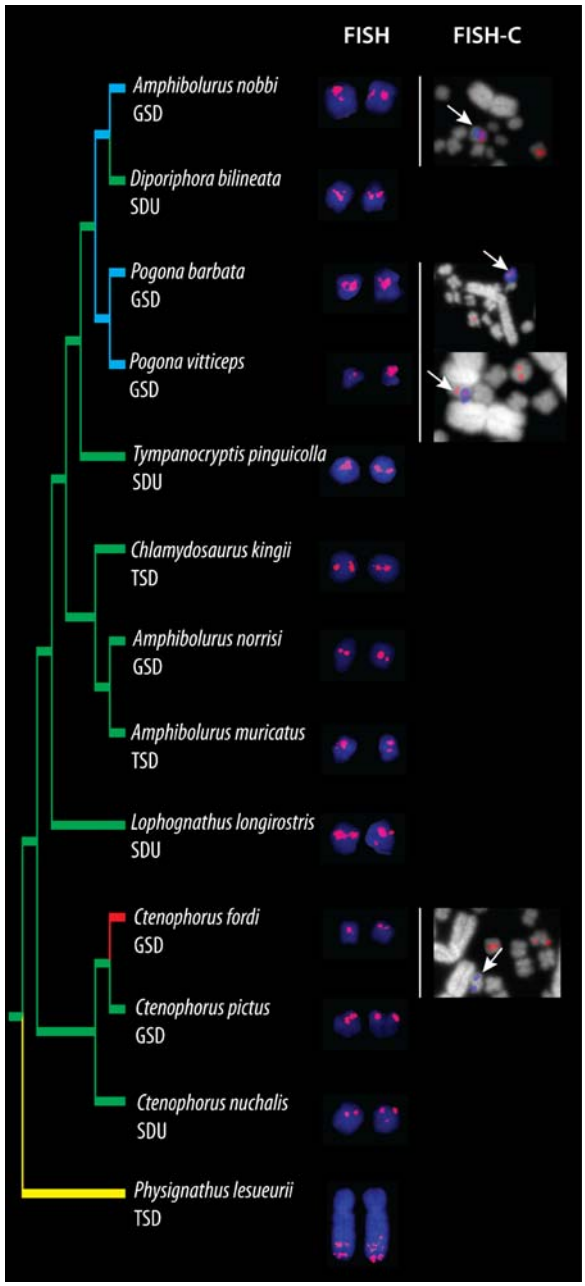

Fig. 2 Phylogeny of dragon lizard species included in this experiment showing physical mapping of PvZW3 probe in 13 species and subsequent FISH and C-banding in four species (pruned phylogenetic tree modified after Hugall et al. 2008). Where PvZW3 hybridization is concordant with C-banded microchromosomes, the branch is indicated by blue; red indicates a discordant microchromosomal location; and yellow indicates PvZW3 hybridized to a macrochromosome. Arrows indicate C- banded W chromosomes; red hybridization signals represent location of PvZW3; SDU, sex determination unknown. Images were captured, analysed, pseudo-coloured and merged (including C-bands in merged C-FISH images) using IPlab (Scanalytics Inc., Virginia, USA)

we could not detect sex-specific C-bands, but we identified a highly heterochromatic sex microchromosome in *Pogona barbata, A. nobbi* and *C. fordi*, revealing female heterogamety in these species. This follows our identification of a W microchromosome in *P. vitticeps* (Ezaz et al. 2005) and a report of ZW macrochromosomes in the Asian agamid *Phrynocephalus vlangalii* (Zeng et al. 1997). Male heterogamety has not yet been reported in agamids; thus it is possible that in members of the Agamidae that have GSD, female heterogamety is a conserved mechanism, as in birds and snakes (Ohno 1967; Solari 1994). The size of the Z and W chromosomes and the C-banding pattern in these species provide no clues as to whether the sex chromosomes are ancient or evolutionarily recent, but accumulation of repetitive sequences implies at least initial differentiation (Ohno 1967; Bull 1983; Charlesworth 1991; Graves 2006). Deletion events are likely to have been involved in differentiation of the W chromosome. However, the current techniques, particularly on such small sex chromosomes cannot detect such deletions.

The molecular probe PvZW3, derived from the sex microchromosome pair of *Pogona vitticeps*, hybridized to both members of a microchromosome pair in 11 of 12 species examined. Two of these species, *Amphibolurus muricatus* and *Chlamydosaurus kingii*, exhibit TSD, suggesting that they have homologues of the *P. vitticeps* sex chromosomes that do not participate in sex determination. In the third TSD species *Physignathus lesueurii*, the probe hybridized to the tips of the long arm of a macrochromosomal pair, indicating that there has been a chromosomal rearrangement within the Australian agamid lineage involving the PvZW3 sequence. Unlike most of the Australian agamids, which have a diploid chromosome complement 2n=32 (12 macro and 20 micro), *Physignathus lesueurii* has a diploid chromosome number of 2n=34 (12 macro and 22 micro) (Witten 1983), indicating that rearrangements between macro- and microchromosomes have occurred. Subsequent C-banding identified the microchromosome pair with the PvZW3 hybridization signal as the heterochromatic W microchromosome and the putative Z microchromosome in *Pogona barbata* and *A. nobbi*, implying that the Z and W chromosomes of these two species are homologous to those of *Pogona vitticeps*.

Perhaps our most important finding is that the PvZW3 probe hybridized to a microchromosome pair in *Ctenophorus fordi*, which did not include the heterochromatic W microchromosome identified by subsequent C-banding. This suggests that the ZZ/ZW mechanism of sex determination in *C. fordi* involves a microchromosome pair that is different from the ZW microchromosomes of *Pogona vitticeps*, *Pogona barbata* and *A. nobbi*. Either an original ZW system, still present in the *Pogona-A. nobbi* clade, was usurped by a neo-sex-determining gene on a different microchromosome pair in the *Ctenophorus fordi* lineage, or the reverse occurred. Identifying the sex chromosomes in outgroups to these species could distinguish these alternatives. Switches in the sex chromosome pair could also have occurred via an intermediate TSD state in the absence of sex chromosomes. The novel pair of sex chromosomes could have arisen when an allele on an autosomal pair acquired a female-determining function, defining a new W chromosome. Such multiple and independent evolution of novel sex chromosomes is quite remarkable given the apparently short time frame spanning the radiation of the Australian agamids from an Asian ancestor (~25 million years; Hugall et al. 2008), but is not unique; for instance multiple and independent origin of female heterogamety has recently been described in two closely related species of medaka fishes (Takehana et al. 2008).

An alternative explanation is that the ZW sex chromosome pair is conserved within the Australian agamids, but the PvZW3 sequence has been separated from the sex-determining locus by a rearrangement in *C. fordi*. Resolution of the alternatives could be resolved by comparative chromosome painting or gene mapping.

Agamid lizards exemplify the types of shifts between TSD and GSD that have been proposed generally for reptiles, and our data suggest that shifts from one ZW system to a different ZW system may also have occurred within this short time frame. Such a transition has apparently occurred between birds and snakes, which show non-homologous ZW pairs. Further molecular and cytogenetic investigation of the Australian agamid lizards could reveal much about the molecular mechanisms accompanying such changes.

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