Evolution of Reptile Sex Chromosomes

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A thesis submitted for the degree of
Doctor of Philosophy of the Australian National University

Canberra, March 2010

Declaration

Except where specific reference is made to other sources, the work presented in this thesis is the work of the author. It has not been submitted, in whole or in part for any other degree.

Denis O'Meally

Title page image: The first scientific illustration of an Australian snake, *Pseudechis porphyriacus*; from G. Shaw, '*Coluber Porphyriacus* the Crimson-Sided Snake', in *Zoology and Botany of New Holland, and the Isles Adjacent, the Zoological Part*, J. Sowerby, London, 1794, Plate 10.

Abstract

Reptiles and birds (Sauropsida) are a diverse group of organisms that have persisted for more than a quarter of a billion years. The methods used in determining sex are remarkably varied and so provide innumerable opportunities in comparative analyses, over both short and very long evolutionary timescales. I exploit this phylogenetic depth in addressing several questions about sex chromosomes that have broader relevance to understanding the special evolutionary forces that act on these unique genomic regions.

Sex chromosomes arise from an ordinary pair of autosomes by the acquisition of a sex-determining gene. This gives rise to radical differences in the chromosome pair on which the gene(s) responsible for sex determination lie. In snakes, the progressive stages of sex chromosome differentiation can be observed from the homomorphic ZW of pythons to the highly differentiated W chromosome of elapids. I show that W chromosome differentiation is accompanied by an increasingly complex suite of repetitive DNA, the distribution of which coincides with the female-specific region of the W chromosome. Cross-species hybridisation of chicken W chromosome paint demonstrates that repetitive sequences are shared between the sex chromosomes of birds and derived snakes, despite independent origins.

To investigate this further, I constructed a low coverage cytogenetic map of the tuatara, *Sphenodon punctatus,* and built a preliminary integrated map for the tropical clawed toad, *Xenopus tropicalis*. These maps do not support ancestral synteny of bird and snake sex chromosomes, but finer scale mapping may reveal an as yet undetected association. I used published comparative maps to establish for the first time that synteny of snake Z-linked genes has been conserved for at least 166 million years. Antiquity of sex chromosomes and large differences between homologs are thought to be prerequisite features in the evolution of global dosage compensation mechanisms. I examined relative expression levels of seven putative Z-linked genes in male and female Eastern brown snakes, *Pseudonaja textilis*, but found no evidence for such mechanisms in snakes. If dosage compensation does occur, it is likely gene and tissue specific, as in birds.

Reconciling the modes of reptile sex determination with their distribution over the phylogeny makes plain the conclusion that transitions have occurred and new sex chromosomes have evolved many times over. To examine the pace of these transitions, I contributed to a cytogenetic study of amphibolurine agamids. Known as dragon lizards, these species represent a uniquely Australian radiation that exhibit both temperature-dependent sex determination (TSD) and genotypic sex determination (GSD). Hybridisation of a sex chromosome marker

isolated from *Pogona vitticeps* to metaphase spreads from closely related taxa reveals that sex chromosomes have evolved at least twice and that transitions between TSD and GSD have occurred over the last ~25 million years.

In the course of this work, I was closely involved in the development of a new method for establishing tissue cultures from Australian lizards, with which chromosome preparations can be made. The method uses small, non-invasive biopsies that can be collected in the field without the need to sacrifice animals. Such a technique is particularly useful in initiating cell lines from vulnerable or endangered species as it provides a virtually unlimited source of material for cytogenetic, evolutionary and genomic studies. I also contributed to a literature review of the phylogenetic distribution of GSD and TSD in lizards. This review emphasises the evolutionary lability of sex determination in squamates and suggests a close relationship between ZW sex chromosome systems and TSD.

Acknowledgements

I am incredibly grateful to Jenny Graves for taking me on as a student and providing a fantastic learning environment – I could not have imagined a better laboratory in which to undertake a PhD. Jenny's enthusiasm is infectious and the generosity of the Comparative Genomics Group staff reflects this. Their expertise has proved second to none. I'm indebted to Pat Meithke, Jan Elliot, Ke-Jun Wei and to Drs Edda Koina, Amber Alsop, Marg Delbridge, Janine Deakin and Paul Waters for making my introduction to cytogenetics and genomics a smooth and productive one.

Dr Tariq Ezaz has been a terrific mentor and great friend over the last four years. I couldn't have hoped for a more knowledgeable, approachable, generous, understanding, enthusiastic and amiable supervisor. Tariq's technical expertise was at times a lifesaver, and always inspiring. His readiness to encourage my (sometimes wandering) research, accept my suggestions and celebrate novel findings has been invaluable. Despite being the only two reptile workers in the lab, I truly felt part of a team. Our discussions on wide ranging topics were always informative and thoroughly enjoyable. In short, it was a hoot!

Thanks also to my co-supervisors Steve Sarre and Arthur Georges at the University of Canberra, and to the many members of the AERG. Thanks also to Scott Edwards, Dan Janes and the members of the Ornithology Lab at the MCZ for making my short visit there a rewarding one. My thanks also go to Hilary Millar, whose collaboration has been productive and hassle free.

The many students and visitors to the lab, to RSBS and from further afield made Canberra a vibrant and rewarding setting in which to study. Thanks to my fellow students for being terrific friends and making lab-life that much more enjoyable – Shafagh Al-Nadaf, Hannah Bender, Tim Hore, Jason Limnios, Ali Livernois, Nisrine El-Mogharbel, Veronica Murtagh, Hardip Patel, Vidushi Patel, Claudia Rodrigues Delgado, Carly Smith and Rami Stiglec. Thanks also to Kristen Jordan-Rogers, Nerida Harley, Daniel McMillan, ever ready to crack a joke, laugh at one, or whistle a merry tune. Thanks to Paul Waters and Julie Chaumeil for being generous of spirit(s) – or more usually beer – and to Julie for her fine French cuisine. Thanks to Liz Murchison who, through her refusal to leave the bench, inaugurated the CGG Bar. A special thanks to Hardip and Vidushi for the reliable supply of delicious curries.

I could not have covered such a breadth of topics (or species) without the help of many. I am indebted to the following people and organisations for their assistance in obtaining specimens or providing data and generosity with their time. My sincere thanks go to John Cann of La Peruse, Sydney. To Robert Mason, from the DNA laboratory, and Ross Sadlier and Cecilie Beatson from the Herpetology Section of the Australian Museum for access to specimens and being ever convivial and generally helpful. To the staff of the Veterinary and Quarantine Centre and Herpetology section at Taronga Zoo; Steve Donnellan at the South Australian Museum; Kazumi Matsubara at Hokkaido University, Japan; and Amy Sater at Houston University, my sincere thanks. Thanks also to Peter Child from ReptilesInc. in

Canberra for access to his collection; and to Carly Smith and Hannah Bender for their assistance in bloodletting (a reciprocal arrangement with one snake in Hannah's case).

My thanks go to those who assisted with fieldwork to remote places in often very hot or very wet or very cramped conditions (or combinations thereof). First off, thanks to the Vice Chancellor and Jenny Graves for providing money to drive around the Centre and Top End. Thanks to Paul Waters for assistance in the Northern Territory and his fearless approach of snakes. Thanks to Caz Wales, Bryan O'Meally and Paul Cheshire for driving more than 10,000kms in two weeks from Sydney to Victoria, to Uluru and back via Byron Bay, in January! Thanks to the students and staff of the Institute for Applied Ecology at UC for housing and tending to animals prior to my getting to them.

Finally, thanks to my many awesome friends and family back in Sydney. My visits to Sydney, and your occasional visits to Canberra were utter highlights, and something I always looked forward to. Thanks to my parents, Mary and John, for your constant support and encouragement of my academic pursuits from an early age, and especially in the last few months whilst writing-up. Thanks to my brothers Patrick, Michael and Bryan for being tops. Thanks to Andy, Arch, *Bosom*, Caz, Chesh, Dave, Helen, Hugh, Jim, Kate, Lisa, Meta, Mike, Tiff and Wiz for your interest in my work and willingness to provide light (and often heavy) relief from the pressures of study.

Papers published

O'Meally, D., et al, (in prep.) Evolution of amniote sex chromosomes. *Trends in Genetics*

O'Meally, D., et al, (submitted) Non-homologous sex chromosomes of birds and snakes share repetitive sequences. *Chrom Res*

O'Meally, D., et al, (2009) The first cytogenetic map of the tuatara, *Spenodon punctatus*. *Cytogen Gen Res* **127:** 213-223

Ezaz, T., Sarre, S., **O'Meally, D.**, et al. (2009) Sex chromosome evolution in lizards: independent origins and rapid transitions. *Cytogen Gen Res* **127:** 249-260

Ezaz, T., Quinn, A.E., Sarre, S., **O'Meally, D.**, et al . (2009). Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. *Chrom Res* **17**:91-98

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O N E - Introduction

Sex and the multitude of ways in which it is determined throughout the natural world have been subjects of philosophical enquiry for millennia (Mittwoch 2000). The dichotomous outcome of sex determination – male or female – belies a complex evolutionary history and an enormous variety in the mechanisms different organisms employ. In some animals this decision is made after fertilisation by environmental cues, such as ambient temperature at a critical period in embryonic development (Bull 1983). In some species, sex is decided at fertilisation by heritable factors. Sex chromosomes were first described more than a century ago (McClung 1901) and are now known to have evolved independently in all manner of animals and even some plants (Solari 1994; Ainsworth 2000). These cues provide the initial signal (*viz.* the primary sex determinant) to a complex regulatory network of genes and hormones that ultimately – and reliably – produces either male or female offspring.

Long before the discovery of primary sex determining genes, biologists recognised that in many species, sex is under the control of discrete factors inherited in a Mendelian fashion. Only two days short of a year since Mendel first presented his seminal work to the Natural Sciences Society of Brünn (and thirty-five years before its English translation in 1901) it seems this was appreciated by Darwin. In clarifying the "non-blending of varieties", he wrote in a letter to Wallace, dated 6 February 1866:

> *I crossed the painted lady and purple sweet peas, which are very different coloured varieties, and got even out of the same pod both varieties, perfect, but none intermediate...Though these cases are in appearance so wonderful, I do not know that they are really more so than every female in the world producing distinct male and female offspring.* (Darwin 1866)

In most mammals and medaka fish, a single gene on the Y chromosome (*SRY* and *DMY*, respectively) exerts a dominant effect so that its presence directs sexual differentiation down the male pathway (Sinclair et al. 1990; Matsuda et al. 2002). In birds, it appears that the same developmental fate is sealed not by presence or absence of a gene but by the number of copies (dosage) of the sex-linked *DMRT1* (Smith et al. 2009). Candidate genes have been identified in some other taxa but their role as the primary sex determinant awaits confirmation. In reptiles with temperature-dependent sex determination (TSD) sex is decided by one or two heritable thermal thresholds, one side of which produces males and the other females. In some lizards, parthenogenesis is not uncommon but usually arises in hybrids (Kearney et al. 2009); in facultative cases, sex is determined by the particular meiotic aberration involved. This short summary provides a largely complete sketch of our current understanding of primary sex determination in vertebrates.

Reptiles and birds (Sauropsida) are hugely diverse and account for more than three-quarters of all living terrestrial vertebrates (Figure 1). They are an incredibly successful group, having persisted for more than a quarter of a billion years and today occupy all regions of the globe. Importantly, the methods they employ in determining sex are remarkably varied, rival the diversity found in fish and eclipse that in mammals. Some employ a single pair of chromosomes that may or may not be differentiated, others use multiple chromosomes; some use temperature and in others, temperature overrides chromosomes. In many squamate families, in some genera and even within one species, more than one mode of sex determination operates. Such variety provides countless opportunities in adding to our understanding of sex determination.

Figure 1. Reptiles comprise an incredibly diverse group. Including birds, they account for approximately three quarters of all land vertebrates and are found throughout all tropical and temperate regions of the world. They inhabit all continents and flourish in all manner of environments. Some, such as the green sea turtle (*top left*), inhabit marine environments; some have taken to gliding across rainforest canopies, like *Draco* (*top centre*) from Southeast Asia. Most inhabit the world's arid regions, like *Diporiphora* (*bottom left*) from inland Australia. Reptiles epitomise the variety in sex determination mechanisms. Many use temperature to determine sex, such as the tuatara (*bottom right*) and many others use chromosomes. Parthenogenesis is not uncommon: in some species it occurs sporadically, as in the Komodo dragon (*bottom centre*), yet others produce clonal, all-female populations that reproduce asexually. With the sequencing of the *Anolis* (*top right*) genome and the availability of BAC libraries from several species, reptiles have truly entered the 'genomic era'.

Reptiles have had a venerable history in the study of sex determination. Ohno's (1967) apposite theory of sex chromosome differentiation was developed in large part by observations he made on the sex chromosomes of snakes. Repetitive DNA sequences with analogues in the

heterogametic sex of plants and innumerable animals were originally isolated from snakes (Singh et al. 1976; Epplen et al. 1982). This same class of repeats (known as *Bkm*, for the snake from which it was first characterised, the banded krait, *Bungarus fasciatus*) even enjoyed a fleeting stint as a candidate universal primary sex determinant (Singh & Jones 1982; Chandra 1984). Their unique phylogenetic position and the variation outlined above makes reptiles particularly useful in studies of the evolution of sex chromosomes and dosage compensation (Modi & Crews 2005). With the availability of large insert genomic libraries from several species, sequencing of the chicken, zebra finch and *Anolis* genomes (and the possibility of many more), reptiles have truly entered the 'genomic era'. The stage is set for a renewed focus on reptiles and the contributions they can make to our understanding of genome biology.

This chapter provides an overview of tetrapod diversity and relationships with an emphasis on reptiles. Next, the evolutionary processes involved in the origin of sex chromosomes and their differentiation are reviewed. Recently, several comparative maps of reptilian sex chromosomes have become available, shedding light on the evolutionary history of amniote sex chromosomes. These studies are reviewed and evidence for the common ancestry of sex chromosomes among amniotes is examined. Finally, I provide an outline of the manuscripts and published articles that make up the body of this thesis.

Tetrapod phylogeny and diversity

Studies in comparative biology require at their foundation a robust phylogeny with which contrasts of form and function can be made. Understanding the common history of any trait is essential to understanding its origins. To that end, a brief discussion of tetrapod relationships is warranted. Emphasis is significantly biased towards groups germane to this thesis. Relevant species' affinities are highlighted and only extant taxa are reviewed. Most Orders and Families of modern tetrapods are considered monophyletic. Many of their interrelationships remain unclear, however, because rapid radiations obscure phylogenetic signals among higher taxa. Recent molecular and paleontological evidence has added considerably to our understanding, albeit sometimes at odds with traditional arrangements (Hugall et al. 2007). Some of the more controversial group affinities are discussed. The relationships and divergence dates of the major groups are presented in Figure 3A (see the figure caption, page 7, for sources and a caveat on divergence estimates).

Amphibians

Early tetrapods (land vertebrates) arose from lobe-finned fish (Sarcopterygii) during the Silurian-Devonian, some 430 – 405 million years ago (MYA) (Benton 1990; Müller & Reisz 2005; Hedges et al. 2006). Aquatic relatives of these early antecedents are the freshwater lungfish

and marine coelacanths. The first amphibians enjoyed a terrestrial environment without other vertebrates for some 50-70MY. Modern amphibians (Lissamphibia; about 6,200 species) include the Gymnophiona (caecilians), which diverged from Caudata (salamanders, e.g. *Ambystoma*) and Anura (frogs, e.g. *Xenopus*) about 330MYA. Frogs and salamanders are generally considered each other's closest relative and diverged some 300MYA.

Amniotes

The origin of amniote egg signalled a major evolutionary advance, allowing for the first time reproduction to take place independently of an aquatic environment. This ultimately led to the permanent colonisation of land by vertebrates, some 360-390MYA. Extant amniotes include the Sauropsida (tuatara, squamates, turtles, crocodiles and birds) and the Synapsida (monotreme, marsupial and placental mammals). Affinities of amniote groups have long been based on the fenestration of the temporal lobe of the skull (Osborn 1903). Figure 2 depicts this schematically. Mammals (synapsids) are characterised by a single temporal opening whilst all remaining amniotes, with the exception of turtles, have two (diapsids). In birds and squamates the fenestrae are highly modified so that they bear only a superficial resemblance to the ancestral condition.

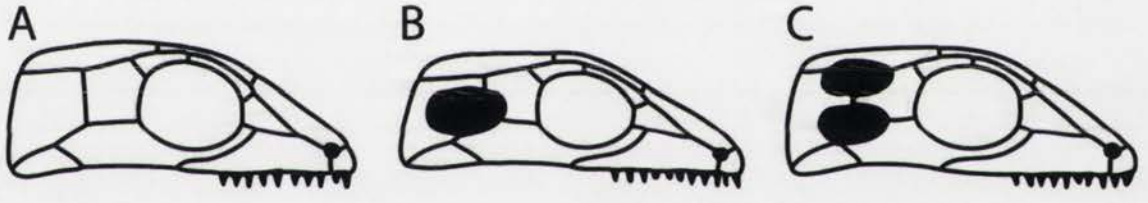


Figure 2. Temporal fenestration of amniote skulls. Exemplars of the (A) anapsid, (B) synapsid and (C) diapsid condition. In extant taxa these generalities are often not apparent and fenestrae are highly modified (figure after Zardoya & Meyer 2001).

Mammals

Synapsida arose early on in amniote evolution and have their origins on the Pangaean supercontinent, in the mid to late Carboniferous (324-310MYA). Extant mammals (Mammalia; about 4,500 species) arose more than 140 million years later, in the mid Jurassic, 166MYA (Bininda-Emonds et al. 2007). Platypus and echidnas are the only living members of Prototheria, and retain a number of pleisomorphic characters with sauropsids, such as a cloaca, oviparity, some skeletal features, and syntenic blocks of genes on sex chromosomes. Metatheria (marsupials) and Eutheria (placentals) diverged from a common ancestor 148MYA. Note that these dates are considerably younger than some previous fossil and molecular data have suggested.

Tuatara & squamates

Lizards, snakes, amphisbaenians and tuatara comprise the extant Lepidosauria, a diverse group with more than 8,500 named species. Tuatara, *Sphenodon*, is the sole representative of a once widespread lineage, Rhyncocephalia, which diverged from early sauropsids 270MYA. The relationships of squamates are not clear and are still hotly debated; however recent studies using nuclear DNA sequence have provided some resolution (Townsend et al. 2004; Vidal & Hedges 2005). Geckoes are among the earliest lizards, having separated some 197MYA, while Scinciformata (skinks and their allies) arose 188MYA. Laterata (amphisbaenians and whiptails) diverged from the remaining squamates (Toxicofera) about 179MYA. This last group includes iguanid lizards, such as the Australian dragons (amphibolurine agamids) and snakes.

Snakes (Serpentes) account for about 30% of extant squamates, and are generally considered to form a monophyletic group. Fossils and molecular data indicate a Gondwanan origin in the Jurassic. A highly derived and simplified body plan coupled with accelerated rates of molecular evolution have conspired to complicate the evolutionary relationships of snakes. Hypotheses on their affinities with other squamates have produced a voluminous literature that does not bear review here. Within Serpentes, many recent phylogenetic analyses have proposed relationships that are quite different from traditional arrangements (and this literature is duly multifarious). Scolecophidea (blindsnakes, literally 'worm snake') are fossorial ant and termite specialists numbering about 370 extant species. They are generally considered to be members of the most basal snake lineage with late Jurassic origins (150MYA). Alethinophidia (the 'true' snakes; about 2,700 species) have traditionally been arranged according their ability to produce venom and its delivery apparatus: pythons, boas and allies representing the most primitive condition, the rear-fanged colubrids intermediate and highly venomous vipers and elapids with their specialised front fangs the most derived.

A profusion of molecular studies suggests that this view is overly simplistic (Vidal & Hedges 2009 and references therein). A detailed review is beyond the scope of this discussion, however some pertinent groupings are consistently obtained. Henophidia (pythons, boas and their allies) diverged 103MYA and remain basal to the Caenophidia ('new' snakes), but pipe snakes and dwarf boas have earlier origins ("Amerophidia"). Vipers appear to have arisen much earlier in the radiation of snakes than previously thought, perhaps 54MYA. Colubridae as traditionally defined is paraphyletic, and most authors now restrict the name to include just five subfamilies (Lawson et al. 2005; Vidal et al. 2007). Among them is Colubrinae, which includes ratsnakes e.g. *Elaphe quadrivirgata*, and treesnakes such as *Dendrelaphis punctulata*. Elapids, including the Australian species *Notechis scutatus* and *Pseudonaja textilis*, diverged from the group including colubrids 46MYA.

Turtles

The position of Testudines (turtles) in the amniote phylogeny has been the subject of debate for well over one hundred years. Such is the derived state of turtles, with their numerous peculiar adaptations and rapid cladogenesis (Shaffer et al. 1997), that their relationships remain uncertain to this day. Testudines has traditionally been considered the sister taxon of diapsids (Sauria) as the only extant member of Anapsida (including several other extinct lineages possessing a single temporal fenestration). Timing of embryonic organogenesis suggests a basal position within sauropsids (Werneburg & Sánchez-Villagra 2009). Others have proposed that they are basal to all amniotes (Gaffney 1980); however, more recent analytical methods, molecular and paleontological evidence have seriously challenged (debunked) this hypothesis. Cladistic analysis of morphological characters suggests affinities with the Sauropterygia, extinct marine reptiles whose extant relatives are lepidosaurs (squamates and tuatara) (Debraga & Rieppel 1997). Analyses of protein, mitochondrial and nuclear DNA sequences suggest that turtles are most closely related to archosaurs (birds and crocodiles) (Caspers et al. 1996; Cao et al. 2000; Iwabe et al. 2005; Hugall et al. 2007). Furthermore, combined analyses of 118 vertebrate gene families support this view (Cotton & Page 2002). Developmental, morphological and molecular data then, suggest that the anapsid condition of turtles is derived, a view that is accepted here.

Extant turtles (~320 species) are divided into two widely accepted groups. The side-necked turtles (Pleurodira) have a typically Gondwanan distribution; today they are found in Australia, Africa and South America. This group includes the Australian Eastern long neck, *Chelodina longicollis*. Turtles that bend their neck vertically (Cryptodira) have a worldwide distribution and include softshell, box, marine and land turtles. Included in this group is the Chinese softshell, *Pelodiscus sinensis*. The two groups have their origins in the late Triassic about 207 MYA.

Crocodiles and birds

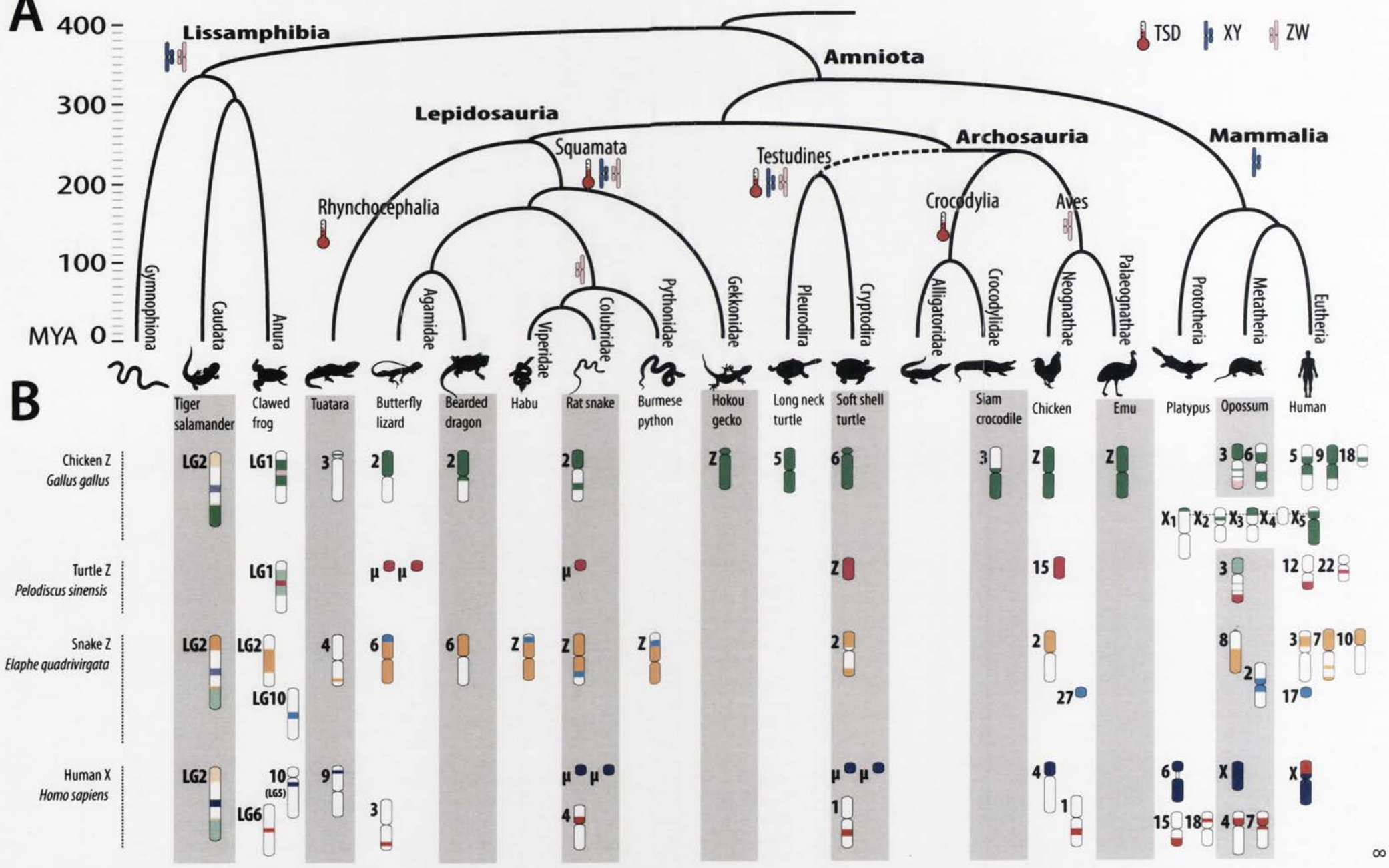
Archosauria includes all living birds (more than 9,500 species), crocodilians and their extinct relatives, such as dinosaurs. Early archosaurs are well represented in the fossil record, and the bird-crocodile divergence is frequently used to calibrate molecular clocks at 245MYA (e.g. Rest et al. 2003; Hugall et al. 2007); however, 'independent' (some calibration points are shared) molecular estimates vary by more than ±40 million years.

Three main groups of living birds (Neornithes) are recognised, although the antiquity of each and within-group relationships are still hotly debated (Ericson et al. 2006; Brown et al. 2007; Hackett et al. 2008). Paleognathae includes ratites (ostrich, emu, kiwis, rheas and allies) and

tinamous from Central and South America. This earliest lineage diverged in the Cretaceous, 120MYA. Neognathae is comprised of Galloanserae (waterfowl and game fowl, including the chicken, *Gallus gallus*) and Neoaves (all other birds; about 95% of all species). Estimates of the Galloanserae-Neoaves split vary widely, but most suggest a mid-Cretaceous origin 105MYA.

Crocodile-like animals first appeared in the Mesozoic and reached their greatest diversity in the Cretaceous. Their tendency to inhabit riparian environments has led a substantial fossil record. Extant crocodilians (Crocodylia) are rather less speciose: about 23 species are found throughout the tropics and subtropics. Alligatoridae (alligators and caimans) and Crocodylidae (crocodiles and allies) first appear in the late Cretaceous, while Gavialidae (the piscivorous Indian and false gharials) arose somewhat later. Paleontological and molecular divergence dates are widely discordant: estimates range from 150-50MYA for the Alligatoridae-Crocodylidae split, and 70-30MYA for Crocodylidae-Gavialidae.

Figure 3 (following page). **Phylogeny of tetrapods and amniote sex chromosome homologies.** **(A)** A consensus tree of tetrapod relationships, indicating the distribution of sex determination modes and sex chromosome systems. Branch lengths are proportional, with divergence dates from http://timetree.org (Hedges et al. 2006), Hedges and Kumar et al. (2009) and authors therein, or as cited otherwise in the text. "Timetree" dates are weighted means of published molecular estimates or determined by expert review (Hedges & Kumar 2009). There is considerable controversy about the approach of Hedges et al. (e.g. Graur & Martin 2004) and their dates are used here provisionally. Only representative taxa are shown to give some indication of the depth of divergence in the major groups. The uncertain placement of turtles (Testudines) is indicated by a dotted line. Alternative topologies place turtles sister to lepidosaurs or closer to birds than crocodiles. They are traditionally placed basally with respect to sauropsids. MYA: millions of years ago; XY: male heterogamety; ZW: female heterogamety; TSD: temperature-dependent sex determination.
(B) Generalised representation of chromosome and linkage group homologies of the chicken Z chromosome (*green*); the turtle *Pelodiscus sinensis* Z chromosome (*magenta*); the snake *Elaphe quadrivirgata* Z chromosome (*yellow* & *cyan*); and the human X chromosome (*blue* & *red*). There is little evidence of an ancestral syntenic association of snake, bird and mammal sex chromosomes, but the *Pelodiscus* Z almost certainly derives from the same ancestral chromosome that gave rise to the gecko and bird Z and platypus X_5. Finer scale gene mapping in reptiles and amphibians may reveal as yet undetected ancestral chromosomal associations. Mapping and painting data are from http://ensembl.org; Shetty et al. 1999; Matsuda et al. 2005; Matsubara et al. 2006; Mikkelsen et al. 2007; Smith & Voss 2007; Kawai et al. 2007, 2009; Uno et al. 2008; Ezaz et al. 2009; Srikulnath et al. 2009a, b; Kawagoshi et al. 2009; *Chapter 4*: O'Meally et al. 2010. *Xenopus* linkage group data are courtesy of Amy Sater (University of Houston) with assistence from Hardip Patel (ANU). LG: linkage group; μ: unidentified microchromosome.


A
400
300
200
100
MYA 0
Lissamphibia
Amniota
Lepidosauria
Archosauria
Mammalia
Squamata
Rhynchocephalia
Testudines
Crocodylia
Aves
Gymnophiona
Caudata
Anura
Agamidae
Viperidae
Colubridae
Pythonidae
Gekkonidae
Pleurodira
Cryptodira
Alligatoridae
Crocodylidae
Neognathae
Palaeognathae
Prototheria
Metatheria
Eutheria
TSD
XY
ZW
B
Tiger salamander
Clawed frog
Tuatara
Butterfly lizard
Bearded dragon
Habu
Rat snake
Burmese python
Hokou gecko
Long neck turtle
Soft shell turtle
Siam crocodile
Chicken
Emu
Platypus
Opossum
Human
Chicken Z
Gallus gallus
Turtle Z
Pelodiscus sinensis
Snake Z
Elaphe quadrivirgata
Human X
Homo sapiens

Sex chromosome evolution in Amniotes

Vertebrates have a bewildering array of sex determination methods, ranging from environmental sex determination (ESD) to genotypic sex determination (GSD) (Bull 1983; Solari 1994; Sarre et al. 2004). While traditionally thought of as mutually exclusive and fundamentally different mechanisms (Bull 1983; Valenzuela et al. 2003), there is strong evidence that the two systems are intimately linked by common evolutionary processes (Sarre et al. 2004; Quinn et al. 2007; Radder et al. 2008; Georges et al. 2010; *Chapter 6*: Ezaz et al. 2010). Reconciling the modes of vertebrate sex determination with their distribution over the phylogeny makes plain the conclusion that transitions between GSD and ESD have occurred many times over. This is as true for higher taxa (Bull 1983; *Chapter 6:* Ezaz et al. 2010) as it is at lower taxonomic ranks (*Chapter 7:* Ezaz et al. 2009). GSD occurs when sex is determined by a constitutional difference in the genotype of males and females. This seemingly innocuous distinction gives rise to radical differences in the chromosome pair on which the gene(s) responsible for sex determination lie. Sex chromosomes spend different amounts of (evolutionary) time in males and females which results in very different evolutionary trajectories. In this section I present an overview of the natural history of sex chromosomes: how they arise, the special evolutionary forces that shape them and consider the possibility of amniote sex chromosomes sharing a common ancestry.

Differentiation of sex chromosomes

In many species with genotypic sex determination, no chromosomes are easily identifiable as the sex pair because they are cytologically homomorphic. In mammals (and some turtles, lizards, frogs and fish), males possess heteromorphic sex chromosomes, where one chromosome is highly degenerated, heterochromatic and gene poor. In this case, they are known as X and Y (XX female: XY male). In all snakes and birds (as well as some turtles, lizards, frogs and fish), the female produces gametes with different sex chromosomes. In this case, the chromosomes are known as Z and W, and the W is similarly depauperate in genes (ZW female: ZZ male). The distinction here is nomenclatural: orthology (or lack thereof) is not inferred by the notation and transitions between XY and ZW systems do occur (Ezaz et al. 2006; Uno et al. 2008). Similar evolutionary forces act in both systems and for the following discussion, Y/W and X/Z are used interchangeably.

GSD comes about by the acquisition of a sex-determining allele at a locus on an ordinary pair of autosomes (Muller 1914, 1918). Differentiated sex chromosomes are thought to arise because of sexually antagonistic alleles (those which are advantageous to only one sex) at loci closely linked to the sex-determining region (Fisher 1931; Bull 1983; Rice 1987b). Selection favours their restriction to one sex by local suppression of recombination which may subsequently spread along most or all of the chromosome (Nei 1969; Charlesworth &

Charlesworth 1980; Bull 1983; Rice 1996). A similar outcome can be effected without suppressed recombination by the evolution of regulatory mechanisms that ensure sex-limited expression of sexually antagonistic alleles; however there are very few examples (Rice 1987b). In the absence of recombination, the nascent Y is inherited clonally and genic decay ensues by the synergistic action of selective processes including 'Muller's ratchet' (Charlesworth 1978), genetic hitchhiking (Rice 1987a), background selection (Charlesworth 1996), as well as drift and mutational overload (Rice 1996). The contribution of each is difficult to dissect experimentally; however Muller's ratchet probably operates more strongly in nascent sex chromosomes and genetic hitchhiking can have profound effects when only a few essential loci remain on the Y (Charlesworth 1996; Hughes et al. 2010).

It is generally agreed that suppression of recombination is the requisite first step in sex chromosome differentiation (Charlesworth et al. 2005), though the mechanism by which this occurs is not well understood. Ohno (1967) reckoned it could be initiated by chromosomal rearrangements such as pericentric inversions; these can occur on the nascent X or Y with equal effect (Charlesworth & Hartl 1978). In *Drosophila* at least, there is genetic variation for site-specific recombination on which selection can act (see Rice 1987b).Others have postulated that asynchronous replication of the sex pair is sufficient to reduce crossing-over (Ray-Chaudhuri et al. 1971). Gorelick (2003) suggests that differential methylation of the sex determining region on the nascent Y can initiate suppressed recombination and canalise its degeneration. Detailed examination of evolutionarily recent sex chromosome systems, such as those presumed in some flies, fish, frogs and dragons, are essential to understanding the initial stages of differentiation (e.g. Bachtrog et al. 2008; Charlesworth 2004; Yoshimoto et al. 2008; *Chapter 7*: Ezaz et al. 2009).

Several studies examining divergence between gametologs (relic genes on Y or W chromosomes and their X- or Z-borne partners) in humans, mice, cats, cows, birds and plants suggest some common features of reduced recombination (Lahn & Page 1999; Sandstedt & Tucker 2004; Pearks Wilkerson et al. 2008; Van Laere et al. 2008; Nam & Ellegren 2008; Bergero et al. 2007). In both XY and ZW systems, restriction of recombination proceeds in a gradual, stepwise fashion and imparts a signature of discrete evolutionary strata along the length of the chromosome, the origins of which can be dated (although see Hughes et al. 2010, and the discussion on page 14). In some systems, recombination suppression has preceded intrachromosomal rearrangements of the X or Z that subsequently led to a disruption of the ancestral gene order (Sandstedt & Tucker 2004; Nam & Ellegren 2008). In humans, it appears that recombination suppression has occurred in concert with large intrachromosomal inversions of the Y chromosome (Lemaitre et al. 2009). Where ancestral gene order is

preserved, progressively older strata are located further from the pseudoautosomal region (a region of the sex chromosomes present in some taxa where meiotic paring and recombination still occur).

The addition of autosomal segments to sex chromosomes leaves similarly characteristic evolutionary signals and provides a mechanism to buffer the degenerative effects of clonal inheritance on heteromorphic chromosomes (Graves 1995, 2006). Comparative mapping and chromosome painting in therian mammals, for example, has revealed that the human X is composed of an ancient 'conserved' and a recently 'added' region (Wilcox et al. 1996; Graves 1998; Glas et al. 1999). The X ancient region is common to all therian mammals, while the added region is autosomal in marsupials. Further evidence of this comes from chicken and other basal vertebrate lineages, in which the two segments map to two or more different autosomes (Figure 3B). In humans, the Y chromosome is composed almost entirely of the added region, and the ancient region has largely been lost due to the degenerative processes so far described (Waters et al. 2001). Autosomal additions that by chance contribute sexually antagonistic genes may be favoured by selection, further enhancing the genetic isolation of the neo-sex chromosomes and accelerating the degeneration of the neo-Y (Rice 1996; Charlesworth et al. 2005). Additional evidence of neo-Y formation comes from the many diverse taxa that possess multiple sex chromosome systems, which must arise because of partial or complete autosomal fusions with sex chromosomes (Bull 1983).

Evolution of dosage compensation

Highly degraded sex chromosomes pose a problem for the species in which they occur. In the homogametic sex, X-borne genes are present in two copies or doses, as is the case for autosomal loci. Where wholesale genic decay has occurred on the Y chromosome, sex-linked genes are present in only one dose. Autosomal anuploidy usually has dire developmental if not lethal consequences in most organisms, but this is not so for sex chromosomes. In many species, compensatory mechanisms have evolved to restore the balance between sex-linked genes and autosomes. Mechanisms have also evolved that ensure transcriptional equality between sexes.

Dosage compensation mechanisms are best characterised in taxa with male heterogamety, namely *Drosophila*, nematodes and mammals. They exemplify three different solutions to the same problem. In male (XY) *Drosophila*, transcription of the single X is up-regulated so that its dose is equivalent to autosomes, and that in females (XX) where both Xs are transcribed (Marín et al. 2000). This is achieved through a male-specific riboprotein complex that binds the X chromosome, alters chromatin structure and effectively doubles its transcription (Straub &

Becker 2007). In worms and mammals (man and mouse at least), X chromosomes are up-regulated approximately two-fold by an unknown mechanism, so that transcription is equivalent to autosomal loci in XO or XY males (Gupta et al. 2006; Nguyen & Disteche 2006). Without some countermeasure, this would leave females (XX) effectively (transcriptionally) tetraploid for X-linked loci. In hermaphrodite (XX) worms a complex of eight proteins (DCC) modifies X chromatin so that transcription from each is reduced approximately two-fold (Meyer 2005). In female therian mammals, one X is silenced (paternal or random X inactivation) so that transcriptional equality between the hyperactive X and autosomes is restored (Lyon 1961; Sharman 1971). Interestingly, X inactivation is not complete, suggesting that some dosage differences are tolerable in females (Carrel & Willard 2005; Heard & Disteche 2006).

Chromosome wide ('global') dosage compensation mechanisms have long been thought a necessary evolutionary response to (or even involved in) sex chromosome differentiation and gene loss (e.g. Charlesworth 1978, 1996; Rice 1987a, Marín et al. 2000; Engelstadter 2008). It would seem intuitive then, that the problems caused by a gene-poor Y are equally grave for a gene-poor W chromosome. Recent data from birds, silkworms and snakes, in which female heterogamety is the norm, suggest that this is not the case (Itoh et al. 2007; Ellegren et al. 2007; Zha et al. 2009; *Chapter 2*).

The absence of a global dosage compensation system in birds has been suspected for some time. They have no equivalent of the Barr body; the Z chromosomes replicate synchronously in males and both are transcribed (Cock 1964; Schmid et al. 1989; Kuroda et al. 2001; Kuroiwa et al. 2002). Early allozyme and later qPCR data on the activity/expression of Z-linked genes suggested that some loci were compensated, and others not (Kuroiwa et al. 2002; McQueen et al. 2001; Baverstock et al. 1982). The same is true of silkworms, and for many other Lepidoptera (Suzuki et al. 1998, 1999; Koike et al. 2003; Traut et al. 2007). These early results have now been convincingly confirmed by broad scale microarray studies that measured global expression ratios of males:females (Itoh et al. 2007; Ellegren et al. 2007; Zha et al. 2009). In chicken, zebra finch and silkworms, the vast majority of genes on the Z chromosome are not subject to dosage compensation in males. Some genes are specifically up-regulated in males, and local or gene-specific down-regulation also occurs (Melamed & Arnold 2007; Melamed et al. 2009; Zha et al. 2009). This probably comes about by augmenting existing regulatory networks and in chickens at least, compensated loci are associated with CpG islands and CR1 transposable elements (Mank & Ellegren 2008; Melamed & Arnold 2009; Zha et al. 2009). In female chickens, the expression ratio of Z-linked to autosomal genes ranges from 0.7 in brain to

0.9 in heart, suggesting that some dosage compensation occurs between sex-chromosomes and autosomes (Itoh et al. 2007).

Among amniotes, chromosome-wide dosage compensation is only known in therian mammals, but the mechanisms involved differ between placentals and marsupials (Heard & Disteche 2006; Deakin et al. 2009). In both groups, however, the inactive X is condensed and late replicating in female somatic cells. Monotremes, like birds, undergo partial dosage compensation, but not all X chromosomes replicate asynchronously in females (Deakin et al. 2008; Ho et al. 2009). Early studies of snake Z chromosomes suggest they replicate synchronously in males (Bianchi et al. 1969). In *Chapter 2*, I provide preliminary evidence that dosage compensation is gene and tissue specific in snakes. So it seems that some form of dosage compensation occurs in all amniote lineages with relatively old, differentiated sex chromosomes, but it is only global in therian mammals. This cannot be due to the age of the sex chromosomes. The snake ZW originated 100-166MYA (*Chapter 3*); the bird ZW at least 120MYA; the platypus 5X5Y later than 166MYA and the therian XY 148MYA, with later modifications in eutherians (Figure3A B). Nor can it be due to the size or number of genes on the sex chromosomes: in therian mammals, the X makes up only 3-5% of the haploid genome; the five platypus X chromosomes account for about 15%; and the Z chromosomes of both snakes and birds about 7-10% (Beçak et al. 1964; Ohno 1967; Bianchi et al. 1969; Graves 1987; Deakin et al. 2008).

In chickens and silkworms, compensated genes have different functions from those that are not, suggesting that dosage compensation has evolved for individual genes according to selective pressures on each (Melamed & Arnold 2007; Zha et al. 2009; Mank & Ellegren 2008). Overall, Z-linked genes are over-expressed in males and many of these may have male specific functions. *DMRT1* is a notable case in chickens as dose is thought to determine sex (Smith et al. 2009); the same is true for many Z-linked genes in silkworms (Zha et al. 2009). If the selective advantage of over-expression to males outweighs the disadvantage to females (given some Z:autosomal compensation occurs), there will be no selective pressure to evolve a global hyper-transcription and subsequent down regulation system as found in mammals and nematodes (Naurin et al. 2010). Because Y/Z/X/W chromosomes spend different amounts of evolutionary time in males (respectively 100/67/33/0%), higher male mutation rates and sexual selection may lead to faster gene loss from the Y chromosome and greater selective pressure to evolve global dosage compensation (Naurin et al. 2010). Others have suggested that mammalian X inactivation has evolved because of maternal-paternal antagonism, and that a role in dosage compensation is secondary (e.g. Haig 2006). Non-model species such as

platypus and snakes are well placed to test these and other novel hypotheses on the evolution of dosage compensation.

Repeat accumulation on degenerate sex chromosomes

In the absence of recombination, sex-specific chromosomes are subject to massive amplification of repetitive sequences. The most notable of these are simple, highly repetitive satellite sequences, often found in tandem arrays or in large palindromic structures that arise by ectopic recombination and gene conversion (known as 'ampliconic' repeats); and retrotransposons (reviewed in Charlesworth et al. 1994). Studies in *Drosophila* suggest that retrotransposons are likely responsible for the characteristic accumulation of heterochromatin on the degenerated sex chromosome (Steinemann & Steinemann 1998). Heterochromatin may act as a cellular defence against retrotransposon invasion, mediated by RNA-interference based silencing and associated with genetic inertness and late replication of the Y chromosome (Steinemann & Steinemann 2005; Steinemann et al. 1993). Differential condensation of the X and Y chromosomes may also be adaptive as it prevents crossing-over that could result in potentially lethal recombination products (McKee & Handel 1993).

Most sex chromosome repeats tend to be amplified in a species-specific manner, even in closely related taxa. This suggests rapid repeat amplification and molecular differentiation occur repeatedly after divergence from a common ancestor. Recent sequencing of the male-specific euchromatic region of the Y chromosome (MSY) in human and chimpanzee has provided a detailed picture of the remarkable changes that have occurred since their divergence 6MYA (Skaletsky et al. 2003; Hughes et al. 2010). The MSY of both species has undergone significant rearrangement, such that the structure in a common ancestor cannot be inferred. The rate of gene loss (and transpositional gain) differs markedly, chimps having lost one third of the distinct genes or gene families and half the protein coding transcription sites with respect to humans. Hughes et al. (2010, p538) assert this is "more comparable to the difference in autosomal gene content in chicken and human, at 310 million years of separation". Ampliconic repeats are also represented differentially in the human and chimpanzee MSY. The chimp MSY has several unique palindromic structures and greater numbers of those that are common to humans. Overall, these repeats account for roughly equal proportions of the MSY in humans (53%) and chimps (57%) but in absolute terms, are 44% larger in chimpanzees. In each species, more than 30% of MSY sequences have no homologous counterpart in the other. Of the sequences can be aligned, identity is comparable to autosomal loci (98.3% vs 98.8% at autosomal loci). This may explain the gradual changes suggested by gametolog analyses (Lahn & Page 1999; Lemaitre et al. 2009) compared to the dynamic changes revealed by full sequence analysis (Hughes et al. 2010).

Some repeats have a broader taxonomic distribution and can be used to infer the ancestral organisation of sex chromosomes. Lineage specific amplification suggests stochastic processes play an important role. *ZMB*-related repeats occur along the length of the W chromosome in the zebra finch and homologous sequences are autosomal or Z-linked (Itoh et al. 2008). Southern blot surveys of other birds show that these repeats are found across Neognathae, but are only sex-specific in closely related songbirds (Passeriformes). This suggests that *ZMB* was present before the radiation of neognathous birds, but was amplified only on the W of zebra finch and its close allies. Similar repeats have been isolated from other birds, such as *P2000-17* from the lesser black gull (Griffiths & Holland 1990); *Apa*I family repeats were originally isolated from galliform birds, but are amplified on the W of all neognathous birds (Yamada et al. 2006).

A third class of sex chromosome repeats has an even wider taxonomic distribution, and occurs in the heterogametic sex of both plants and animals (Jones & Singh 1981; Singh et al. 1981; Arnemann et al. 1986; Schäfer et al. 1986; Nanda et al. 1990, 1991; Parasnis et al. 1999). The banded krait minor-satellite (*Bkm*) was first isolated from snakes and consists of tandem arrays of GATA and GACA sequences (Singh et al. 1976; Epplen et al. 1982). *Bkm* is often, but not always associated with heterochromatin both in autosomes and degenerate sex chromosomes (Jones & Singh 1985; Nanda et al. 1991). In snakes, its frequency in the genome corresponds with the degree of ZW differentiation (Jones & Singh 1985; *Chapter 3*). While a common origin of *Bkm* repeats has been assumed (e.g. Epplen et al. 1983), the simple sequence and tandem structure is prone to amplification by slipped-strand mispairing and similar replicative errors, so independent origins are equally likely (Epplen 1988). Proteins that specifically bind *Bkm* have been isolated from the gonads of snakes, silkworms, mice and man (Singh et al. 1994; Priyadarshini et al. 2003). A role in regulating the decondensation and transcriptional activation of Y or W heterochromatin has been suggested but is yet to be demonstrated (Singh et al. 1994).

Sex chromosome degeneration has a phylogenetic pattern

Snakes provided Ohno (1967) with the material on which he based his theory of how sex chromosomes differentiate from autosomal pairs. He and his co-workers supposed the progressive stages in sex chromosome degeneration could be observed among the representative families of snakes (Beçak et al. 1964) (Figure 4).

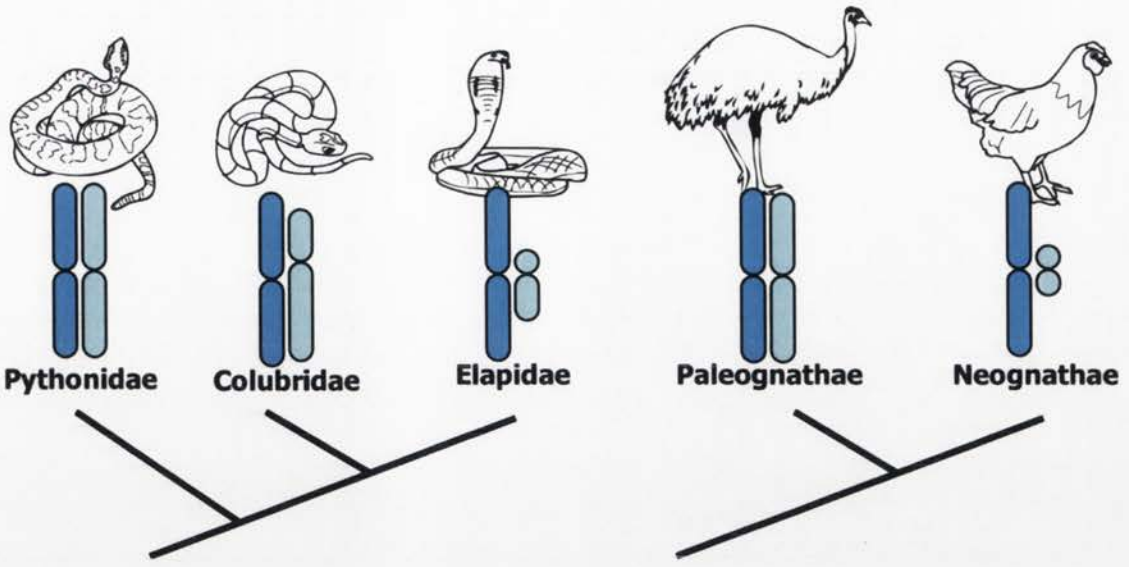


Figure 4. W chromosome degeneration has a distinct phylogenetic pattern. This pattern was first recognised in the various families of snakes by Beçak et al. (1964) and later in birds by other workers. Pythons and paleognathes have virtually homomorphic sex chromosomes, while more derived lineages including elapid snakes and neognathous birds, such as the chicken, possess highly differentiated sex chromosomes. A similar pattern has been reported in invertebrates, fish and mammals. *Light blue* represents the W chromosome and *dark blue* the Z.

Pythons and boas represent the extant members of the most basal snake lineage whose cytology has been examined extensively (blind snakes, Scolecophidia, remain largely uncharacterised). With the exception of one species, pythons and boas possess homomorphic sex chromosomes that are cytologically indistinguishable (Mengden & Stock 1980; Olmo & Signorino 2005). Members of the Colubridae (a mostly non-venomous and morphologically 'intermediate' group; see discussion page 5) are characterised by sex chromosomes that are often similar in size but differ in centromere position or the extent of heterochromatin. Members of the Elapidae and Viperidae (independently derived snake lineages that are often highly venomous) possess markedly dimorphic sex chromosomes which may differ greatly in size, centromere position and heterochromatin. In some elapids at least, the appearance of the heteromorphic chromosome can differ substantially between even closely related species (Mengden 1982), suggesting rapid change after speciation.

Comparative maps of snake sex chromosomes have shown that this trend is reflected in the genic content of the snake W chromosome (Matsubara et al. 2006). In *Python molurus*, eleven genes and their relative order on the W chromosome correspond exactly to those on the Z chromosome, although the W is possibly smaller in size. The W chromosome of the colubrid *Elaphe quadrivirgata* is about 25% smaller than the Z and it bears only three of the eleven Z genes mapped. The crotaline viper *Protobothrops* (syn. *Trimeresurus*) *flavoviridis* possesses the

most extreme differentiation of the sex pair: none of the eleven Z genes maps to the W chromosome. The order of gene loss and a conserved ZW telomeric repeat indicate that degeneration of the W chromosome proceeded from the short arm.

The same progressive stages of W chromosome degeneration have been described in palaeognathous birds and between palaeognathous and neognathous birds (Takagi et al. 1972; Graves & Shetty 2001; Tsuda et al. 2007). Within Paleognathae, ratites possess a near homomorphic ZW pair but tinamous (the sister group of ratites) have a ZW pair that differs in gene content and the extent of repeat accumulation. The Z and W of neognathous birds are usually highly differentiated (there are some notable exceptions, e.g. de Oliveira et al. 2005). Analysis of gametologs indicates that degeneration of tinamou W chromosomes and those of neognathous birds occurred independently (since the Paleognathae-Neognathae split), but in parallel (de Kloet & de Kloet 2003; Tsuda et al. 2007; Mank & Ellegren 2007). Analogous phylogenetic patterns of sex chromosome degeneration have been described in mammals (Graves 2006) and fish (Kirpichnikov 1981, cited in Rice 1996). In some extreme cases, degeneration may proceed to such an extent that it ultimately leads to the total loss of Y or W chromosomes (reviewed in Graves 2002, 2006).

Conversely, the great unanswered question about sex chromosome degeneration is why some presumably old Ws or Ys *don't* do it. Two models have been proposed, both of which assume a primary role for Muller's ratchet in sex chromosome decay. In some (perhaps most) plants, genes that are expressed in the diploid adult stage are equally active in the relatively long-lived, haploid gametophytes, and therefore subject to selection (Charlesworth 1978; Gorelick 2005). This renders such plants immune to Muller's ratchet as any deleterious alleles will be lost, rather than being sheltered by non-deleterious alleles on a homologous chromosome. Occasional sex reversal could also render Muller's ratchet ineffective if recombination between XY or ZW pairs occurs in sex-reversed individuals (Perrin 2009). Rearrangements and sequence heterology between the X and Y are common in taxa with highly differentiated sex chromosomes, preventing recombination in sex-reversed individuals. This is not so in amphibians and reptiles in which structurally differentiated sex chromosomes are uncommon and occasional sex reversal occurs, even under natural conditions. This model could be particularly applicable to basal snakes and ratites, although sex reversed individuals have not yet been reported (no simple molecular test of sex exists for snakes). Interestingly, many pythons brood their eggs and some generate warmth by shivering (Harlow & Grigg 1984; Mierop & Barnard 1978). Most adaptive explanations of this behaviour cite increased offspring survival as a result of paternal care. A corollary could be the buffering of a thermosensitive sex

determination system prone to genotypic sex-reversal. Anecdotal reports suggest sex determination may be thermosensitive in pythons but it has not been tested empirically.

The evolutionary history of amniote sex chromosomes

There is no doubt that sex chromosomes have evolved independently many times in amniotes, given their haphazard distribution across the phylogeny (Solari 1994; Ezaz et al. 2006; Organ & Janes 2008; *Chapter 6*: Ezaz et al. 2010; *Chapter 7*: Ezaz et al 2009). Indeed, the apparent lack of homology between the sex chromosomes of amniotes is frequently taken as evidence of their independent origins from different ancestral autosomes (Nanda et al. 2002; Matsubara et al. 2006; Graves 2008). An alternative – but compatible – view is that the sex chromosomes of major groups were ancestrally syntenic, and arose by independent rearrangements in some lineages.

Smith and Voss (2007) find support for this model in the large chromosomes of the tiger salamander, *Ambystoma tigrinum*. They mapped a limited number of chicken Z and 2 and human X orthologs to the same chromosome (linkage group 2) in the salamander, which last shared a common ancestor with amniotes some 360-390MYA (Figure 3A, B). This implies that the sex chromosomes of snakes, birds, monotremes and therian mammals all derive from the same ancestral autosome, albeit with sex under the control of a different gene (or genes) in each case. Smith and Voss find further support for this scenario by comparing amniotes with teleost outgroups. *Chapter 3* reports results showing that the chicken W shares repetitive sequences with the snake W, despite 275 million years of independent evolution (Figure 3A). It is noteworthy also, that the chicken Z chromosome shares homology with four of the five platypus X chromosomes (Veyrunes et al. 2008).

Two recent studies have used *in silico* approaches in reconstructing the ancestral vertebrate karyotype. Neither came to this same conclusion; however, nor did they address the question of ancestral synteny of amniote sex chromosomes explicitly (Kohn et al. 2006; Nakatani et al. 2007). An integrated linkage, physical and sequence map for the tropical clawed toad, *Xenopus tropicalis*, is nearing completion, and has been made available to interested researchers (A. Sater, U Houston pers. comm.). This map provides an independent test of the conclusions of Smith and Voss (2007).

Early workers hypothesised the homology of bird and snake Z chromosomes because they are morphologically quite similar (Beçak et al. 1964). Not only are the Z chromosomes of each often metacentric (alternate centromere positions are now known); they are similar in absolute size, accounting for about 10% of the haploid genome; and in relative size, being the

fourth or fifth largest chromosome in most snakes and birds. However, recent mapping data show clearly that the snake and bird Z chromosomes are not homologous (Figure 5).

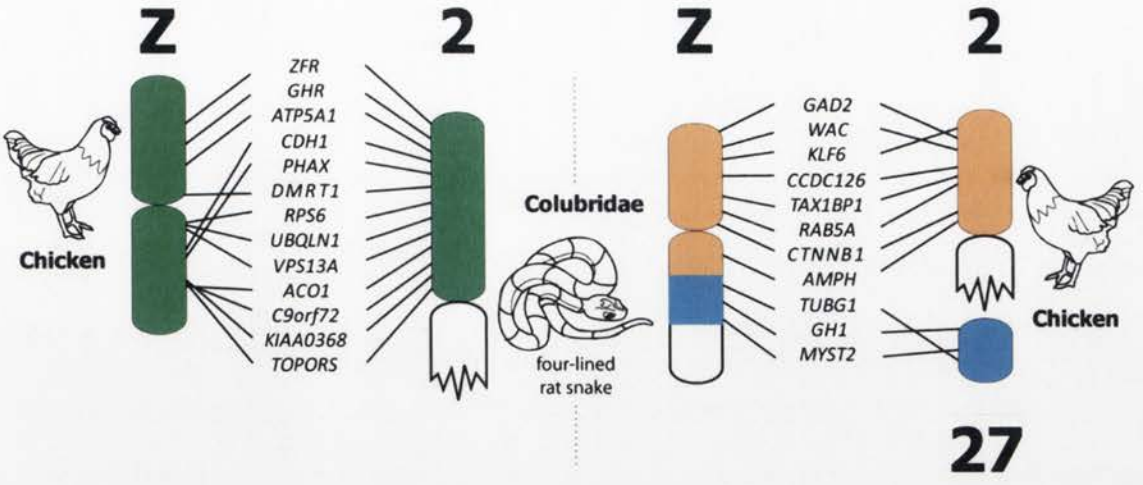


Figure 5. Comparative map of the snake *Elaphe quadrivirgata* and chicken sex chromosomes. The snake Z is composed of genes found on chicken chromosomes 2 (*yellow*) and 27 (*cyan*). The chicken Z (*green*) is equivalent the short arm (and a small region of the long arm, not shown) of chromosome 2 in the snake. Mapping data are after Matsuda et al. (2005), Matsubara et al. (2006) and Kawai et al (2007).

The snake Z is homologous to the short arm of chicken chromosome 2 and chromosome 27 (Matsuda et al. 2005; Matsubara et al. 2006; Kawai et al. 2007), and to chromosome 6p in the agamid lizards *Pogona vitticeps* and *Leiolepis reevesii* (Ezaz et al. 2009; Srikulnath et al. 2009b; Figure 3B). This suggests that the synteny of snake Z genes has been conserved for 166 million years. The snake Z must have arisen by the fusion of ancestral segments (represented by chicken chromosomes 2 and 27) between 166-275MYA because these regions are always found on separate chromosomes in other amniotes and on linkage group (LG) 2 and LG10 in *Xenopus*. Neither the low coverage map of the tuatara (*Chapter 4*: O'Meally et al 2010) nor the preliminary *Xenopus* map demonstrates ancestral synteny of snake and bird Z-linked genes as they each correspond to different chromosomes or linkage groups (Figure 3B; *Chapter 3*).

Comparative mapping of sex linked genes in birds and mammals also dispelled early notions of homology between the bird Z and human X (Fridolfsson et al. 1998; Graves & Shetty 2001; Nanda et al. 2002). The chicken Z corresponds to regions of human chromosomes 5, 9 and 18 and opossum chromosomes 3 and 6 (Figure 3B). The opossum X and the human X conserved region correspond to chicken chromosome 4. The added region of the human X corresponds to chicken chromosome 1. The chromosomal segments representing Z and X are not syntenic in any other amniote so far examined.

Clearly an amniote outgroup (amphibian or fish) is required to determine if these regions were syntenic in an amniote ancestor. Teleost fish such as *Danio*, *Fugu* and *Oryzias* could fit the bill but they are not ideal for two reasons: the teleost genome duplication complicates detection of orthologs (Taylor et al. 2001) and, probably *because* of the duplication, their genomes are rearranged and synteny is poorly conserved (Nakatani et al. 2007). The preliminary *Xenopus* map lends no support to the conclusions of Smith and Voss (2007). The chicken Z chromosome is homologous to *Xenopus* LG1; the snake Z is homologous to LG2 and LG10 and the human X is homologous to LG6 and chromosome 10 (=LG5) (Figure 3B).

In some cases *Xenopus* linkage groups share more orthologs between amniote sex chromosomes than would be expected by chance (that is, given an even distribution of orthologs along a chromosome; see *Chapter 3* for a more detailed analysis). This may indicate the presence of as yet undetected ancestral syntenies, but finer scale mapping and analyses are required. The 'pre-duplication' genomes of the dogfish, lamprey and sea urchin, when fully assembled and mapped will help resolve these competing hypotheses.

There may still be a case for a common origin of amniote sex chromosomes, albeit with a different focus. The chicken Z represents a unique ancestral vertebrate chromosome. Synteny of the genes it bears has been maintained to a greater or lesser extent in all extant tetrapods (Figure 3B). Using novel computational methods, Nakatani et al (2007) were able to reconstruct hypothetical karyotypes for the pre-duplicated genome of the ancestral vertebrate; after one round of genome duplication in the ancestral gnathostome; and after two rounds in the ancestral amniote. Remarkably, synteny of the ancestral vertebrate chromosome 'A' is conserved in the gnathostome chromosome 'A0'; the amniote chromosomes '3' and '26'; and ultimately chicken chromosomes Z and 17; spanning a period of time in excess of 500 million years.

This same ancestral chromosome also has an unusual tendency to become (remain?) a sex chromosome. The Z chromosome of the chicken is shared by all birds, although there are some lineage specific rearrangements. In the bizarre platypus sex chromosome system of five X and five Y chromosomes, the same syntenic block is represented, mostly by the second largest X chromosome (Veyrunes et al. 2008). Gene mapping in the basal squamate *Gekko hokouensis* (Hokou gecko) also reveals conserved synteny and gene order in the Z and slightly degenerated W with the chicken Z chromosome (Kawai et al. 2009).

The Z chromosome of the Chinese softshell turtle, *Pelodiscus sinensis*, shares synteny of 5 genes with chicken chromosome 15, suggesting origins in a different autosomal pair of chromosomes than the chicken Z (Kawagoshi et al. 2009). The chicken Z chromosome is

homologous to chromosome 6 in this species; though the gene order is different (Matsuda et al. 2005). Chicken 15 and Z share no synteny in the hypothetical ancestral amniote karyotype of Nakatani et al. (2007), but they do in the hypothetical tetrapod and vertebrate karyotypes proposed by Kohn et al (2006). In opossum and *Xenopus* these 5 genes (and most within their bounds) are syntenic with regions that are orthologous to chicken Z (Figure 3B). This is strong evidence for a common evolutionary origin of the turtle and bird (and gecko and platypus) sex chromosomes in the tetrapod ancestor.

Chapter summary and aims

This thesis presents the results of my research in the format of a number of manuscripts and publications. The appendix lists oral presentations and reproductions of posters given at local and international conferences. Broadly, the objective of this thesis was to examine the mechanisms of sex chromosome evolution in reptiles using Australian snakes and lizards as model groups. In particular, the aims were:

Aim 1: *To investigate the status of dosage compensation in snakes*

Chapter Two describes a preliminary study on dosage compensation in a derived Australian elapid snake whose W chromosome is a degenerated relic of the Z.

Aim 2: *To characterise the molecular aspects of sex chromosome degeneration in snakes*

Chapter Three discusses the evolution of sex chromosome differentiation in snakes including evidence of repetitive sequences shared by the apparently non-homologous sex chromosomes of birds.

Aim 3: *To investigate the synteny of amniote sex-linked genes in basal reptile lineages*

Chapter Four presents the first cytogenetic map of the tuatara and discusses the dynamics of genome organisation and evolution in this unique and archaic reptile. (In press)

Aim 4: *To develop a straightforward method for making chromosome preparations for cytogenetic studies in reptiles*

Chapter Five describes a new method for establishing tissue cultures from Australian lizards that obviates the need for sacrificing animals. (Published)

Aim 5: *To determine what, if any, factors predispose changes in mechanisms and modes of sex determination*

Chapter Six reviews the literature on sex chromosome evolution lizards, emphasising their lability and suggests a close relationship between ZW sex chromosome systems and temperature-dependent sex determination. (In press)

Aim 6: *To investigate the tempo of sex chromosome turnover and modal changes in sex determination systems*

Chapter Seven presents work on the sex chromosomes of Australian agamid lizards in which sex determining mechanisms have evolved independently at least twice over the last 25 million years. (Published)

The results of these studies and future research avenues are summarised in Chapter Eight.

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T W O - Dosage compensation in snakes

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Current status of paper (circle as appropriate):

Planned/In Preparation/Submitted/Under Revision/Accepted/Published

Date paper accepted for publication or anticipated date of publication:

2010

Name of journal:

Current Biology or PLoS Genetics

Extent to which research is your own:

I designed the experiments, collected samples, performed all laboratory work and conducted all analyses.

Your contribution to writing the paper:

I wrote the draft of the paper and prepared figures.

If paper not yet accepted, has the paper been rejected by any journals:

No

Comments:

This chapter reports a preliminary study on dosage compensation in snakes. Although the snake Z chromosome is conserved, genes remain unmapped in our study species. The samples size is low and statistical analysis is rudimentary; efficiencies of some qPCR reactions are sub standard and some primers are awaiting validation by sequencing of products. This chapter follows the format of a manuscript submission, and so figures are presented at the end of the chapter rather than in the text; the materials and methods section refers to published protocols rather than reporting them in detail.

Dosage compensation in snakes

Denis O'Meally

Abstract

Many female snakes have differentiated ZW sex chromosomes, in which the W chromosome is heterochromatic, rich in repetitive sequences and depauperate of genes. This leads to a dosage imbalance between the genes on sex chromosomes and autosomes and between the sexes. In *Drosophila*, *C. elegans* and mammals, global compensatory mechanisms have evolved to restore such imbalances. To date, no studies have explicitly examined the dosage compensation status of sex-linked genes in snakes. Here, we show that the W chromosome of the Eastern brown snake, *Pseudonaja textilis* (Serpentes: Elapidae), is almost entirely female-specific and rich in repetitive sequences. We used RT-qPCR to determine relative expression of seven putative Z-linked genes in males and females. Equivalent male and female expression in liver but male biased expression in brain and spleen suggests that dosage compensation is tissue specific, and some genes appear to be specifically regulated. Although differences between male and female expression are generally not statistically significant, there is a strong trend towards higher expression in males, suggesting that no general mechanism for dosage compensation exists in snakes.

Introduction

Differentiated sex chromosomes are common in many animals and some plants (Solari 1994; Ainsworth 2000). They spend different amounts of evolutionary time in males and females because of their role in sex determination and consequent inheritance patterns. In species with male heterogamety, such as mammals and fruit flies, males have different sex chromosomes (XY: male & XX: female). In species with female heterogamety, such as snakes, birds and moths, females have a distinct sex pair (ZZ: male & ZW: female). Despite independent evolutionary origins, there are some striking similarities between different sex chromosome systems. The Z and X are usually large and gene rich and are often specialized in gene content (Reinke et al. 2000; Rogers et al. 2003; Lercher et al. 2003; Storchová & Divina 2006). The sex-specific chromosomes, Y and W, are often heterochromatic, highly repetitive and depauperate of genes, leading to different doses of sex-linked genes in males and females (Marín et al. 2000). At autosomal loci, such a situation usually has dire developmental if not lethal consequences (e.g. Lindsley et al. 1972), but this is not so for sex chromosomes.

Many species have evolved systems to restore the dosage balance between genes on sex chromosomes and autosomes, and between the sexes. In *Drosophila*, transcription of the single X in males is upregulated by a male-specific riboprotein complex, so that the dose of X-linked genes is on par with autosomal loci in both sexes (Straub & Becker 2007). In mammals and nematode worms, all X chromosomes are hyper-transcribed so that in males, transcription from the single X is equivalent to that of autosomes (Nguyen & Disteche 2006; Gupta et al. 2006; Lin et al. 2007). In female nematodes, both X chromosomes are downregulated to equal autosomal levels of transcription (Meyer 2005). In female therian mammals, one X chromosome is transcriptionally silenced (known as X-inactivation) to achieve the same outcome (Heard & Disteche 2006; Deakin et al. 2009). These systems operate chromosome-wide and result in balanced expression of autosomal and X-linked genes; and expression ratios of X-linked genes in males and females (M:F) approximately equal to 1.

In other species, dosage compensation appears to occur on a gene-by-gene basis, if at all. At several X-linked loci in female platypus, two alleles are transcribed in 50% of cells but only one is transcribed in the remainder, indicating that transcriptional silencing is incomplete and stochastic (Deakin et al. 2008). M:F expression ratios are less than 0.7 at four of ten X-specific loci, implying no global upregulation. In male chickens, both Z chromosomes are transcriptionally active (Kuroda et al. 2001; Kuroiwa et al. 2002). Only a minority of Z-linked genes are equally expressed in males and females (Itoh et al. 2007). For all Z-linked genes, average M:F expression ratios range from 1.2 in liver to 1.7 in fetal brain, suggesting that no

general mechanism exists to balance dosage inequalities between the sexes (Itoh et al. 2007; Mank & Ellegren 2009). In female chickens, Z-linked genes are transcribed at 0.7-0.9 times the average level of autosomal loci, but in males the range is 0.9-1.1 (Itoh et al. 2007). Chromatin at the Z-linked 460Kb *MHM* locus is hypomethylated and hyperacetylated only in female chickens with the same histone modification present on the single, upregulated X in male *Drosophila* (Bisoni et al. 2005; Teranishi et al. 2001). *MHM* may mediate Z-autosome balance in females by initiating higher transcription from the single Z (Graves 2003; Bisoni et al. 2005; Melamed & Arnold 2007). In silkworms, the average M:F expression of Z linked genes ranges from 1.3-1.5 in the soma to 11.5 in gonads (Zha et al. 2009). Male biased expression in birds and silkworms probably reflects not only the dosage difference, but also the bias in male-specific functions of Z-linked genes (Scholz et al. 2006; Storchová & Divina 2006; Zha et al. 2009).

Dosage compensation has not been examined explicitly in any species of snake. All have female heterogamety but the Z and W chromosomes are not always morphologically distinguishable. Basal lineages such as boas generally possess a cytologically identical ZW pair, colubrid W chromosomes are characterized by simple inversions and heterochromatin accumulation, while vipers and elapids have a highly rearranged, heterochromatic and gene poor W chromosome (Beçak et al. 1964; Mengden 1981; Matsubara et al. 2006). The gene content of the Z chromosome is conserved across species, and it makes up 8-10% of the haploid genome (Beçak et al. 1964; Mengden 1982; Matsubara et al. 2006). Early studies by Bianchi et al. (1969) showed no evidence of asynchrony in replication of the Z chromosomes in male boas, colubrids or vipers. The only evidence of dosage compensation comes from two species of natricine colubrids, in which enzymatic activity of the sex-linked gene *FH* is equal in liver extracts from males and females (King & Lawson 1996). Here we examine the relative expression of seven putative Z-linked genes in the Australian Eastern brown snake, *Pseudonaja textilis*, whose W chromosome is largely female specific, heterochromatic and presumably gene poor. We find no evidence of a general dosage compensation mechanism, but it appears that regulation may be gene and tissue specific.

Materials & Methods

Animals and sample collection

We included in this study four female and three male adult Australian Eastern brown snakes, *Pseudonaja textilis* (Serpentes: Elapidae). We chose this species because it is commonly encountered and the W chromosome is known to be highly differentiated and heterochromatic (Mengden 1981; Skinner et al. 2005). Animals were collected opportunistically during summer

months of 2007-2009. Specimens were sacrificed by intraperitoneal injection of sodium pentobarbital and were dissected quickly after death. Sex was identified post mortem by internal examination of the gonads. Brain, liver and spleen samples were snap frozen in liquid nitrogen and stored at -80°C for later use. We also included an additional liver sample from a female museum specimen (Australian Museum, Sydney EBU4989).

Metaphase chromosome preparation

Chromosomes were prepared from short-term culture of peripheral blood leukocytes or from fibroblast culture according to standard techniques (Ezaz et al. 2009, 2005). Cultures were incubated at 28°C for 3 – 4 days in 5% CO_2. Chromosomes were harvested 2 hours after adding colcemid (75ng/mL) by treating with hypotonic solution (KCl, 0.075mM) and fixed in methanol:acetic acid (3:1) according to standard protocols. The cell suspension was dropped on to slides and air-dried. Slides were kept frozen at -80°C for later use.

Probe preparation and FISH

We used comparative genomic hybridization (CGH) to reveal the female specific region of the W chromosome. Male and female probes were prepared from genomic DNA following the protocol of Ezaz et al. (2005) except that no competitor DNA was used. DNA was extracted using a standard phenol-chloroform procedure (Sambrook & Russell 2001). DNA concentration was measured on a NanoDrop and labeled by nick translation with Orange-dUTP (Abbott Molecular) for female DNA and with Green-dUTP for male DNA. An equal amount of each was used in hybridization experiments. We used synthetic oligonucleotide probes to examine the distribution of *Bkm*-like sequences on the W chromosome. The oligos $(GATA)_4$ and $(GACA)_7$ (Epplen 1988) were conjugated with Cyanine 5 (Geneworks, Adelaide) and resuspended to 1µg/µL in H_2O. Hybridization and microscopy were carried out as previously described (*Chapter 4*: O'Meally et al. 2010).

RNA extraction, primer design and quantitative RT-PCR

Total RNA was extracted from 22 tissue samples using the GenElute Miniprep Kit (Sigma Aldrich) and DNase treated according to the manufacturer's protocol. First-strand cDNA was synthesized by oligo (dT) priming using Superscript III (Invitrogen). Primers for seven putative snake Z-linked genes and a putative autosomal housekeeping gene, *GAPDH*, (Matsubara et al. 2006) were designed by first identifying conserved exonic sequences using Uprobe (Sullivan et al. 2008). RT-PCR was performed using Promega GoTaq Master Mix with initial denaturation at 94°C for 1 min, 35 cycles of 94°C for 20 sec, annealing at temperatures given in Table 1 for 20 sec, extension at 72°C for 1min, and final extension for 10 min. These amplified products were sequenced using ABI BigDye chemistry at the AGRF in Brisbane (see Table 1), also allowing us

to test for biallelic expression in liver of one male (ID# Pt0208-1). From these sequences, species-specific qPCR primers were designed that flank at least one intron, amplify products ranging from 98-136pb, and have similar CG content and melting temperatures (Table 1).

Quantitative RT-PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Amplification and detection was carried out on a Rotorgene 3000 cycler (Corbett Research). Cycling conditions consisted of an initial hold cycle of 95°C for 20 min; 45 cycles of 94°C for 15 sec; annealing at 55°C for 15 sec; and extension and data acquisition at 72°C for 20 sec. Melting curves were constructed from 45°C–95°C to confirm the purity of the PCR products. Relative expression of each gene was determined by normalization to *GAPDH* expression using the comparative quantification module of Rotor-Gene6 software package (Corbett Research). We tested the null hypothesis that there was no difference between male and female relative expression levels using a two-tailed t-test with unequal variances (Welch's t) for each gene and for each tissue.

Results

Comparative Genomic Hybridization and Bkm mapping

We used comparative genomic hybridization (CGH) to reveal the sex specific region of the W chromosome in a female *P. textilis* (Fig 1A). The sex chromosomes are the fourth largest pair and the W is large and submetacentric (Mengden 1981; Skinner et al. 2005). Female specific sequences are found along of the length of the W chromosome, but are concentrated on the subcentromeric region of the short arm. Only the centromere and small telomeric regions are not marked by the probe. We also used *Bkm*-derived probes to examine the accumulation of this simple repeat (Fig 1B, C). The W chromosome shows accumulation of *Bkm* related sequences along its length but the greatest density is found on the distal short arm. Although we did not attempt to map any genes in this species, the high density of repeats suggests that the W chromosome is highly degraded and gene poor.

Biallelic and relative expression levels of putative Z-linked genes

We sequenced products of the first round RT-PCR, both to develop species-specific qPCR primers and to investigate biallelic expression from the Z chromosomes of one male. The sequence totals about 3.6kb over seven Z-linked loci. In all, we found one single nucleotide polymorphism (SNP) at base pair 231 of 608 (3rd codon position) in the amplified *MYST2* fragment, indicating both alleles were actively transcribed in the liver of this individual (Fig 2).

The putative autosomal housekeeping gene *GAPDH* was used to normalize expression levels of seven putative Z-linked genes in brain, liver and spleen. In brain, all genes were expressed at

mean levels greater than *GAPDH* with the exception *TAX1BP1* in males and *WAC* in both sexes (Table 2). With the exception of *CTNNB1* and *KLF6*, all genes were expressed at relatively low levels in liver. In spleen, *RAB5A* in females and *TAX1BP1* and *WAC* in both sexes were expressed at levels lower than *GAPDH*. Relative expression levels less than half that of *GAPDH* are considered here to be below accurate detection limits. Of the genes that are within accurate detection limits, none of the mean expression levels differs significantly between males and females except for *CTNNB1* in spleen (156.67 in male, 96.95 in female; p=0.06, Welch's t). Ratios of male to female expression (M:F) suggest a female bias only for *KLF6* in liver (0.62). In brain, all genes are expressed at higher levels in males (1.24-2.28) as is the case for spleen (1.38-2.28). In liver, expression is equal in both sexes (0.97-1.07). Between individuals, expression levels were highly variable for most genes in brain and spleen. Lower overall expression in liver corresponds with lower inter-individual variation (Figure 3).

Discussion

Dosage compensation mechanisms are thought to evolve because of gene loss on degenerating sex chromosomes (Marín et al. 2000). Such mechanisms should be unnecessary in basal lineages of snakes, as their sex chromosomes are largely homomorphic, each possessing a similar compliment of genes. In derived snake lineages, such as elapids, a selective advantage to dosage compensation is expected because their W chromosomes are highly heterochromatic and presumably gene poor. We used CGH to reveal the female-specific region of the W chromosome in *P. textilis*, which occupies most of its length (Figure 1A). *Bkm* sequences are known to accumulate in the heterochromatic region of snake W chromosomes (Jones & Singh 1985) and their co-occurrence with the female specific probe on the short arm suggests that suppression of recombination was initiated in this region, as for other snakes (Matsubara et al. 2006). Although we have not mapped any genes to the sex chromosomes of *P. textilis*, the degenerated W chromosome and highly conserved synteny of the snake Z (Matsubara et al. 2006; Srikulnath et al. 2009; *Chapter 3*) make this a good candidate in which to examine dosage compensation.

Asynchrony in replication of homogametic sex chromosomes is associated with transcriptional inactivation of one homolog in mammals. X-inactivation serves to restore the balance of hyperactivated X chromosomes between the sexes. The inactive X is late replicating and visible in somatic cells as the heterochromatic Barr body. These are features not so far described in birds or other reptiles (Cock 1964; Schmid et al. 1989; Bianchi et al. 1969). We surveyed seven putative Z-linked genes (~3.6kb of sequence) in one wild caught male and found a single SNP in the transcript of *MYST2*. If, by analogy to X-inactivation, male snakes inactivate one Z

chromosome, this might indicate random inactivation as in therian mammals and some Orthoptera (Rao & Padmaja 1992), or escape from inactivation (Carrel & Willard 2005). However, biallelic expression from this locus, the absence of sex chromatin in males and synchrony in Z chromosome replication strongly suggest that inactivation of one sex homolog does not occur in snakes.

In this preliminary study, we measured relative expression in males and females against a single autosomal control gene, *GAPDH*. Thus, we can calculate expression ratios between the sexes, but not between sex chromosomes and autosomes. In the heterogametic sex of *Drosophila*, *C. elegans*, chickens, primates and rodents, some form of dosage compensation equalizes autosomal expression relative to sex-linked genes in somatic tissues (Gupta et al. 2006; Nguyen & Disteche 2006; Straub & Becker 2007; Lin et al. 2007; Itoh et al. 2007). In some species, such as *Drosophila*, the sex-specific mechanism operates globally, but is poorly understood in nematodes and mammals. In chickens, Z-autosome balance is less effective; it probably occurs on a gene-by-gene basis, but the *MHM* locus may also play a more global role (Melamed & Arnold 2007). The situation in snakes is likely similar; however, more data from autosomal loci are required to address this question.

A previous study of two colubrid snakes suggested dosage compensation might operate because of equal enzymatic activity of the Z-linked *FH* in liver extracts of males and females (King & Lawson 1996). Although we did not measure expression at this locus, our expression data for three genes in liver suggest that M:F expression ratios are generally equal in this tissue (0.97-1.07). Liver also shows relatively low M:F bias in whole-chromosome microarray studies of chicken (1.24) and zebra finch (1.19), species where dosage compensation between sexes is known to be poor (Itoh et al. 2007). Such small biases would be difficult to detect in standard allozyme experiments, so the findings of King and Lawson (1996) may not be strong evidence for dosage compensation in snakes.

In *P. textilis*, all genes show a bias towards male expression in brain (1.24-2.28) and spleen (1.38-2.28), with the exception of *KLF6* (spleen M:F=0.62). *Krupel-like factor 6* is a well-conserved transcription activator and a tumor suppressor gene in humans and mice (Maglott et al. 2005). A sex-specific role in the spleen of female snakes would be speculative and higher female expression may just reflect the low sample size (male-female average expression does not differ significantly; p=0.3, Welch's t). One gene, *TUBG1*, has a M:F expression ratio of 1.24 in brain, which suggests it may be specifically regulated. Although differences between males and females are not statistically significant, male biased expression for the remainder of genes in both tissue types suggests that, as for birds and moths, there is no global mechanism for

dosage compensation between the sexes in snakes (Arnold et al. 2008; Zha et al. 2009). Equal expression between males and females in liver corresponds with lower overall expression of the genes examined therein, but it may also reflect tissue-specific regulation to balance expression between males and females. Larger sample sizes and normalization against different housekeeping genes will establish the veracity of these observations.

Like other ZW taxa such as birds and moths, it seems snakes lack a global mechanism to compensate for gene dosage between the sexes (Traut et al. 2007; Arnold et al. 2008; Zha et al. 2009). Snake sex chromosomes are 105-166 million years old (*Chapter 3*) and the Z accounts for about 10% of the haploid genome, so there should be sufficient evolutionary time and pressure for chromosome-wide mechanisms to develop (Marín et al. 2000), but they apparently have not. Several authors have suggested that the evolution of dosage compensation is mediated by specialized gene content on sex chromosomes and that sexual selection plays an important role (e.g. Naurin et al. 2010; Mank 2009). Homologous syntenic blocks are well conserved throughout Sauropsida (Matsuda et al. 2005; Srikulnath et al. 2009), so changes in the gene content of the snake Z can be inferred over evolutionary time. For example, it would be of interest to see if genes with female-specific functions are underrepresented on the snake Z, as is the case in birds (Storchová & Divina 2006), and if female-specific regulatory changes or autosomal translocations of such genes are coincident with W chromosome degradation. These and other analyses will remain intractable, however, until non-mammalian species are better represented in public genome projects.

Acknowledgements

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) under permits and licenses issued by the New South Wales government (S12364) and with the approval of the Australian National University Animal Experimentation Ethics Committee (Proposal R.CG.07.3b/04/07). We are grateful to John Cann and to Ross Sadlier and Robert Masson (Australian Museum, Sydney) for providing specimens.

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Tables and Figures

Table 1. RT-PCR and RT-qPCR primers used in this study. We initially designed primers for seven snake Z-linked genes and an autosomal control *GAPDH* (Matsubara et al. 2006; Srikulnath et al. 2009) by identifying conserved exonic sequences across vertebrates using Uprobe (Sullivan et al. 2008). After sequencing these products, we designed a second set of nested primers suitable for use in qPCR that are specific to *Pseudonaja textilis*. The columns list the forward and reverse sequences, the annealing temperature (Ta) for each primer pair; the product size obtained and Genbank accession numbers. * For *GAPDH*, the same primer pair was used for quantitative PCR as for the initial round RT-PCR.

	Forward Sequence (5′→3′)	Reverse Sequence (5′→3′)	Ta °C	Product Size	Genbank Accession
First round PCR primers					
CTNNB1 1a2b	CCTGGTTCGATACTGACCTGTA	CCCATAGGAAACTCAGCTTGGT	51	140	XX123456
KLF6 1a2b	AGGCAACTGGGTAGGGTGCAAA	AAGCCTTACAGATGTTCATGGG	55	278	XX123456
MYST2 2a3b	TTGGCCGCTATGAACTGGATAC	CCTCTTTGGCTATCCATTCATC	52	608	XX123456
TUBG1	TGGCCAACCACACCAATCAA	TCAACACAAATTAAGAAGGGCAACTG	51	417	XX123456
RAB5A 2a3b	AGCTGCTTTTCTAACCCAAACT	GTTGTGTGGGTTCAGTAAGGTC	51	429	XX123456
TAX1BP1 1a2b	TGAAGGAAACTCTGACATGCTG	CTAACCTTTTGCTCTGTTTGCA	50	184	XX123456
WAC 1a2b	TACACATGGCTTCCCATGGCTT	CCATCAAACCAGTGGTTCATCC	55	1461	XX123456
GAPDH 1a2b*	GGCACTGTCAAGGCTGAGAACG	TGGAGTCCACTGGTGTCTTCAC	51	119	XX123456
Nested qPCR Primers					
CTNNB1	TGCATTGTGATTGGCCTGTA	GGAAACTCAGCTTGGTTAGTGTG	55	98	XX123456
KLF6	GACCTCGAAGCCTCTTAGCC	CTGTGACAGGTGCTTCTCCA	55	105	XX123456
MYST2	CCTCTCTCAGACTTGGGACT	GCTGTCTCTTGGCTGATCTC	55	119	XX123456
TUBG1	TCTGAGCCCTCCAACCAACACAA	GGGCTTCTCTGTGGCTTACCTCA	55	120	XX123456
RAB5A	TGGTCAAGAACGGTACCACA	TGCTTGCCTCTGGAGTTCTT	55	136	XX123456
TAX1BP1	ACAAAGGCTGGACTTCTTGAGTT	TCAACTTGATCTCTGAGTTGCATT	55	116	XX123456
WAC	TGATTTGGACCAGGAGATGG	AGTCAACGTCTCAGCAGCCT	55	106	XX123456

Table 2. Average relative expression levels of putative Z-linked genes in *Pseudonaja textilis*. Values are normalized against *GAPDH* expression and were determined for four females and three males using total RNA extracts of brain, liver and spleen, with an additional liver sample from one female. Mean values for male expression are significantly different from female expression only for *WAC* in brain (**, p=0.03; Welch's t) and *CTNNB1* in spleen (*, p=0.06). Ratios of male to female (M:F) expression approximately equal to 1 indicate a compensated locus, while ratios greater than 1.4 are consistent with no dosage compensation. Ratios less than 1 indicate female biased expression. Mean values below 0.5 and their M:F ratio (shown in *blue*) are below accurate detection limits.

		CTNNB1	KLF6	MYST2	TUBG1	RAB5A	TAX1BP1	WAC
Brain	Male	132.33	48.00	4.87	2.92	2.97	0.05	0.81
	Female	93.85	27.75	3.48	2.36	1.68	1.03	0.35
	M:F	1.41	1.73	1.40	1.24	1.77	0.05	**2.28
Liver	Male	6.70	1.57	0.23	0.72	0.17	0.03	0.06
	Female	6.88	1.46	0.32	0.72	0.17	0.01	0.05
	M:F	0.97	1.07	0.73	1.00	0.97	3.39	1.39
Spleen	Male	156.67	41.73	3.83	293.83	2.53	0.10	0.75
	Female	96.95	67.70	2.78	128.94	0.90	0.03	0.47
	M:F	*1.62	0.62	1.38	2.28	2.81	3.26	1.60

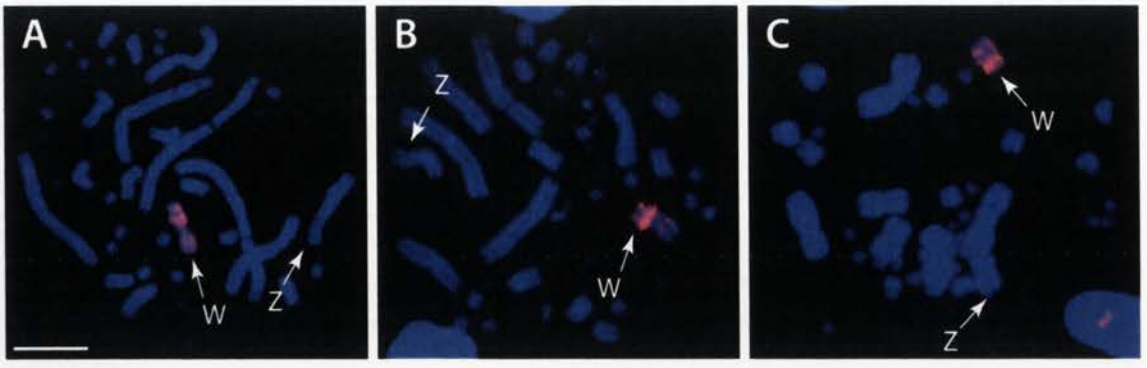

Figure 1. (A) Comparative genomic hybridization of male and female genomic DNA on female metaphase chromosomes of *Pseudonaja textilis*. Female-specific sequences make up the majority of the W chromosome. **(B)** Hybridization of $(GATA)_7$ and **(C)** $(GACA)_4$ *Bkm* probes to female metaphase chromosomes. *Bkm* sequences map along the length of the W chromosome but are concentrated on the short arm. The W chromosome is highly heterochromatic and the high repeat content suggests it is depauperate of genes. The scale bar represents 10μm.

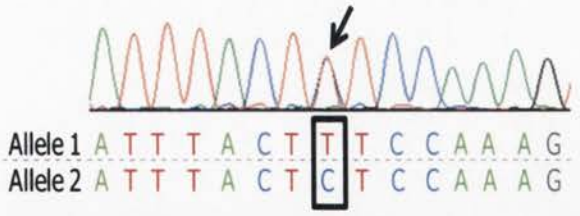

Figure2. Biallelic expression from the putative Z-linked gene *MYST2* in a wild caught male *Pseudonaja textilis*. Expression of both alleles suggests that one Z chromosome is not transcriptionally silenced, in contrast to the inactive X chromosome in female therian mammals.

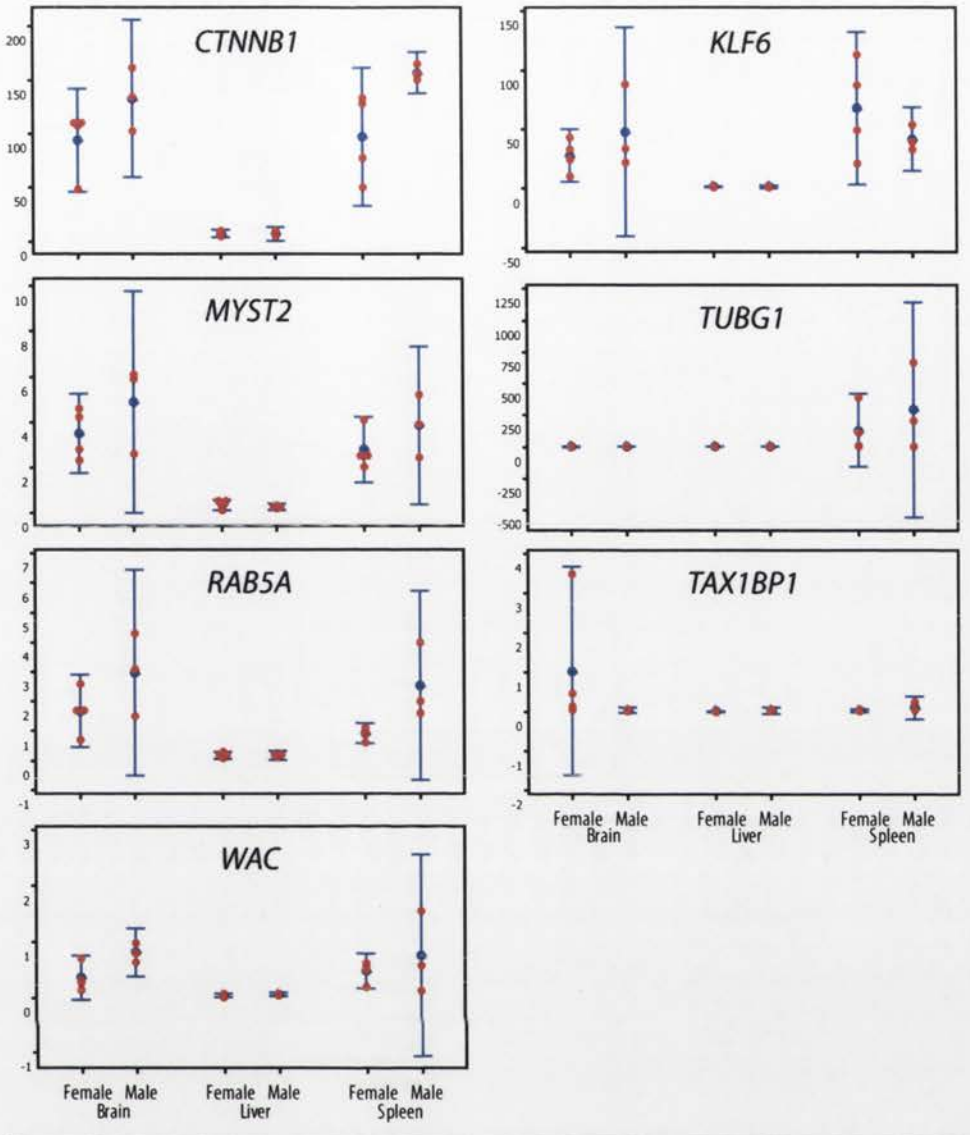

Figure 3. Relative expression levels of seven putative Z-linked genes in brain, liver and spleen from four female and three male *Pseudonaja textilis* (and an additional liver sample from one female). Red dots indicate individual observed expression levels normalized against expression of the autosomal gene *GAPDH*. Blue dots indicate the mean value with 95% confidence intervals indicated in blue.

T H R E E - Non-homologous sex chromosomes of birds and snakes share repetitive sequences

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Current status of paper (circle as appropriate):

Planned/ In Preparation /Submitted/Under Revision/Accepted/Published

Date paper accepted for publication or anticipated date of publication:

2010

Name of journal:

Chromosome Research or Chromosoma

Extent to which research is your own:

I designed the experiments, collected samples, performed all laboratory work and conducted all analyses.

Your contribution to writing the paper:

I wrote the draft of the paper and prepared figures.

If paper not yet accepted, has the paper been rejected by any journals:

No

Comments:

The experiments reported in this paper made use of some existing resources/reagents from previous workers in this laboratory. Bioinformatics support was provided by H. Patel. Amy Sater (U Houston) provided data on *Xenoups* scaffolds and their corresponding linkage groups. This chapter follows the format of a manuscript submission, and so figures are presented at the end of the chapter rather than in the text; the materials and methods section refers to published protocols rather than reporting them in detail.

Non-homologous sex chromosomes of birds and snakes share repetitive sequences

Denis O'Meally

Abstract

Snake sex chromosomes provided Ohno (1967) with the material on which he based his theory of how sex chromosomes differentiate from autosomal pairs. Like birds, snakes have a ZZ male:ZW female sex chromosome system, in which the snake Z is a macrochromosome much the same size as the bird Z. However, the gene content shows clearly that the snake and bird Z chromosomes are completely non-homologous. The molecular aspect of W chromosome degeneration in snakes remains largely unexplored. Using comparative genomic hybridization, we identified the female-specific region of the W chromosome in representative species of Australian snakes. In the basal python *Liasis fuscus*, no sex-specific region can be identified, but the W of the colubrid *Dendrelaphis punctulata* shows a large domain on the short arm that consists of female-specific repeats. The large W of *Notechis scutatus* is composed almost entirely of repetitive sequences, including *Bkm* and 18S rDNA related elements. FISH mapping of both simple and complex probes shows patterns of repeat amplification concordant with the size of the female-specific region in each species examined. Mapping of intronic sequences of genes that are sex-linked in both birds (*DMRT1*) and snakes (*CTNNB1*) reveals massive amplification in discrete domains on the W chromosome of the elapid *Notechis scutatus*. Using chicken W chromosome paint, we demonstrate that repetitive sequences are shared between the sex chromosomes of birds and derived snakes. This could be explained by an ancestral but as yet undetected syntenic association of bird and snake sex chromosomes. We also establish that synteny of snake Z-linked genes has been conserved for at least 166 million years; and that the snake Z consists of two conserved blocks derived from the same ancestral vertebrate chromosome.

Introduction

Sex chromosomes come about by the acquisition of a sex-determining allele at a locus on an ordinary pair of autosomes (Muller 1914, 1918). Differentiated sex chromosomes arise because of sexually antagonistic alleles (which are advantageous to only one sex) at loci closely linked to the sex-determining region (Fisher 1931; Bull 1983; Rice 1987). Selection favors their restriction to one sex by suppression of recombination, which may subsequently spread along most or all of the chromosome (Nei 1969; Charlesworth & Charlesworth 1980; Bull 1983; Rice 1996). In the absence of recombination, retrotransposons invade sex chromosomes and tandem repetitive sequences are amplified in the non-recombining region (Charlesworth et al. 1994). Heterochromatin also accumulates, perhaps as a cellular defense against unchecked retrotransposition (Steinemann & Steinemann 2005).

Most sex chromosome repeats are species-specific and highly variable, even between closely related taxa. This suggests rapid amplification and molecular differentiation occur repeatedly after divergence from a common ancestor. Recent sequencing of the male-specific euchromatic region of the Y chromosome (MSY) in human and chimpanzee provides a striking example of the changes that have occurred since their divergence 6 million years ago (MYA) (Skaletsky et al. 2003; Hughes et al. 2010). The MSY of both species has undergone significant rearrangement, such that the structure in a common ancestor cannot be inferred. The chimp MSY has several unique palindromic structures and greater numbers of those that are common to humans. In each species, more than 30% of MSY sequences have no homologous counterpart in the other.

Some sex-chromosome repeats have a broader taxonomic distribution whose lineage specific amplification suggests stochastic processes play an important role, e.g. the *P2000-17*, *Apa*I and *ZMB* repeat families isolated from bird W chromosomes (Griffiths & Holland 1990; Yamada et al. 2006; Itoh et al. 2008). Nucleolus organizer regions (NORs) consist of tandem arrays of 18S, 5.8S and 28S rDNA (Shaw & Jordan 1995). When NORs are located on sex chromosomes, their repetitive nature can lead to massive amplification, as on the W of the Chinese soft shell turtle, *Pelodiscus sinensis* (Kawai et al. 2007). The banded krait minor-satellite (*Bkm*) was first isolated from snakes and consists of tandem arrays of GATA and GACA sequences (Singh et al. 1976; Epplen et al. 1982). *Bkm*-related repeats have since been isolated from the heterogametic sex of plants and many animals (Jones & Singh 1981; Singh et al. 1981; Arnemann et al. 1986; Schäfer et al. 1986; Nanda et al. 1990, 1991; Parasnis et al. 1999). In snakes, the frequency of *Bkm* repeats in the genome corresponds with the degree of ZW differentiation (Jones & Singh 1985).

Snake sex chromosomes provided Ohno (1967) with the material on which he based his theory of how sex chromosomes differentiate from autosomal pairs. Ohno supposed the progressive stages in sex chromosome degeneration could be observed among the representative families of snakes. More basal lineages such as pythons and boas (Henophidia) possess homomorphic sex chromosomes that are cytologically indistinguishable. Colubrid snakes are characterized by sex chromosomes that are often similar in size but differ in centromere position or the extent of heterochromatin. Elapidae and Viperidae generally possess markedly dimorphic sex chromosomes, which may differ greatly in size, centromere position and heterochromatin. A similar phylogenetic pattern has been described in many other taxa, including mammals (Graves 2006), fish (Kirpichnikov, 1981; cited in Rice, 1996), and birds (Graves & Shetty 2001; Tsuda et al. 2007; Mank & Ellegren 2007).

Like birds, snakes possess a ZZ male/ZW female sex chromosome system, in which the snake Z is a macrochromosome much the same size as the bird Z, representing 8-10% of the haploid genome (Bianchi et al. 1969). Such are the similarities in gross morphology that early workers hypothesized that the Z chromosomes of birds and snakes were homologous (Beçak et al. 1964). However, recent mapping data show that this is not so (Matsubara et al. 2006; Kawai et al. 2007). The chicken Z is equivalent to chromosome 2p in snakes. The snake Z is homologous to the short arm of chicken chromosome 2 and to chromosome 27. Synteny of eleven Z-linked genes mapped in *Elaphe quadrivirgata* (Colubridae) is conserved in *Python molurus* (Pythonidae) and *Protobothrops flavoviridis* (Viperidae), indicating that the snake Z is conserved across all species (Matsubara et al. 2006).

Here we examine the molecular aspects of sex chromosome degeneration in snakes. We identify the female-specific region of the W chromosome in representative snake species and show that an increasingly complex suit of repeats accompanies the evolution of W chromosome heteromorphy. With few exceptions, most sex-specific repeats reported to date are restricted to closely related taxa. However, we demonstrate that the W chromosome of derived snakes shares sequences with the sex chromosomes of chickens, despite 275MY of independent evolution. This could be explained by an ancestral but as yet undetected syntenic association of bird and snake sex chromosomes. Alternately, such unusual similarity perhaps indicates functional homology of the repeats, and suggests that convergent degeneration is a general property of sex chromosome evolution.

Materials & Methods

Animals

We included in this study representative and common species of Australian snakes. We chose the water python *Liasis fuscus* (Pythonidae); the green tree snake, *Dendrelaphis punctulata* (Colubridae); and the common tiger snake, *Notechis scutatus* (Elapidae). The sex chromosomes of these species show, respectively, various degrees of structural differentiation, ranging from homomorphic to strongly heteromorphic (Shine & Bull 1977; Mengden & Stock 1980; Mengden 1982). For the python samples, we were unable to obtain DNA from a male *L. fuscus* and instead used material from the closely related congener *L. olivaceus*. Numbers and collection localities are given in Table 1. We also used female chicken metaphase spreads available in our laboratory as hybridization controls for most probes.

Metaphase chromosome preparation

Chromosomes were prepared from short-term culture of peripheral blood leukocytes or from fibroblast culture according to standard techniques (Ezaz et al. 2009a, 2005). Cultures were incubated at 30°C for 3 – 4 days in 5% CO_2. Fibroblast cultures were established according to the protocol of (Ezaz *et al.* 2009) or were available in our lab. Chromosomes were harvested 2 hours after adding colcemid (75ng/mL) by treating with hypotonic solution (KCl, 0.075mM) and fixed in methanol:acetic acid (3:1) according to standard protocols. The cell suspension was dropped on to slides and air-dried. Slides were kept frozen at -80°C for later use.

Probe preparation and FISH

For comparative genomic hybridization (CGH), we prepared male and female probes from genomic DNA following the protocol of Ezaz et al. (2005) except that no competitor DNA was used. DNA was extracted using a standard phenol-chloroform procedure (Sambrook & Russell 2001). DNA concentration was measured on a NanoDrop and labeled by nick translation with Orange-dUTP (Abbott Molecular) for female DNA and with Green-dUTP for male DNA. An equal amount of each was used in hybridization experiments.

The chicken W chromosome paint was supplied by Farmachrom (Kent, UK) as primary DOP-PCR product (Griffin et al. 1999). We made subsequent amplifications directly incorporating Orange-dUTP in the products by DOP-PCR. Briefly, the reactions were carried out with 200µM of 6MW primer (5'-CCG ACT CGA GNN NNN NAT GTG G-3') (Telenius et al. 1992), 10µL of 5X buffer with MgCl2, 20mM dNTPs and 1U of GoTaq polymerase (Promega) in 50µL. The reaction mixture was cycled at 94°C for 2 min, 35 cycles of 94°C for 30 s, 62°C for 1 min and 72°C for 5 min and a final extension at 72°C for 10 min.

We used synthetic oligonucleotide probes to examine the distribution of *Bkm*-like sequences on snake sex chromosomes. The oligos $(GATA)_4$ and $(GACA)_7$ (Epplen 1988) were conjugated with Cyanine 3 or Cyanine 5 (Geneworks, Adelaide, Australia) and resuspended to 1µg/µL in H_2O.

To map 18S rDNA in *Notechis scutatus*, we used a BAC clone (AGI 329J14) from the tammar wallaby containing 18S rDNA (Haines 2005). The clone was grown in a 15mL overnight culture and BAC DNA extracted using the Promega Wizard Plus SV Miniprep DNA Purification System according to the manufacturer's protocol (with volumes scaled up).

Homologous probes for *DMRT1* (intron 1) and *CTNNB1* (terminal intron) were prepared by long range PCR for hybridization to *N. scutatus* chromosomes. Genomic DNA from both males and females was prepared as above and used as template for the reaction. Primers were designed to anneal to the exons flanking the intron of interest (DMIF & DMIR for *DMRT1* intron 1 and CTNNB1F & CTNNB1R for the terminal intron of *CTNNB1*, see Table 2 for sequences). Amplifications were carried out using the TaKaRa LA PCR kit (Takara Bio Inc, Japan) according to the manufacturer's protocol and recommended cycling conditions. Positive bands were isolated and subject to a second round of PCR to ensure only single products were used for subsequent analyses. For hybridization, we used amplicons derived from a female specimen (ID # 130964).

To map the terminal intron of *DMRT1* we used a lambda phage clone isolated from a female *N. scutatus* genomic library screened for *DMRT1* (Stiglec 2007). The clone was purified after overnight culture in XLI-blue MRA (P2) *E. coli* (Stratagene) according to standard protocols (Sambrook & Russell 2001). PCR was performed to test for the presence of the conserved intronic and intergenic regions described in Brunner et al. (2001) according to the protocol therein.

For intronic PCR products and the BAC and phage clone, purified DNA was labeled by nick translation with Orange-dUTP. For all hybridizations, 200-500ng of labeled DNA and 1 µg boiled gDNA (not used for CGH and *Bkm* hybridizations) were co-precipitated and resuspended in hybridization buffer (50% v/v deionized formamide, 10% w/v dextran sulphate, 2X SSC, 1X Denhardt's solution and 40 mmol/L sodium phosphate). Hybridization, slide preparation, image capture and analysis followed the protocol of O'Meally et al. (2010).

Sequencing of introns

The first intron of *DMRT1* and the terminal intron of *CTNNB1* were amplified by PCR as above, including a secondary amplification of isolated bands. Amplicons were cloned in to TOP10

vector using the TOPO-XL PCR cloning kit (Invitrogen) according to the manufacturer's protocol. Positive clones were identified by restriction analysis. Sequence was obtained using a primer walking strategy. The primers M13 Forward (-20) and M13 Reverse (Invitrogen) were used to obtain initial sequence, from which subsequent primers (Table 2) were designed. Sequencing was carried out using Big Dye chemistry (Applied Biosystems) at the Australian Genome Research Facility (Brisbane). The repetitive content of the introns was examined using RepeatMasker (Smit et al. 1996) and by searching Genbank *nr* by BLAST (Altschul et al. 1990).

Synteny analyses

To determine if the synteny of genes found on the sex chromosomes of snakes is a recent event, we examined the arrangement of these regions in chicken, human, *Xenopus* and zebrafish. We used the same approach to test for ancestral synteny of genes on the Z chromosomes of snakes and chicken. Using the Ensembl BioMart interface (version 56) (http://www.ensembl.org), we obtained the positions of 15777 orthologs with known locations from chicken (WASHU 2.1, May 2006) and zebrafish (Zv8, Dec 2009); 21389 genes from human (GRCh37, Feb 2009) and zebrafish; and 14643 genes from chicken and human. We also made use of the preliminary *Xenopus tropicalis* integrated linkage map (Sater et al., in prep; JGI 4.1, Aug 2005). We were able to assign scaffolds bearing Ensembl *Xenopus*-human orthologs (7841 genes), *Xenopus*-chicken (6158 genes) and *Xenopus*-zebrafish (9927 genes) to *Xenopus* linkage groups. We inferred ancestral synteny of human chromosomes by counting Ensembl *Xenopus*-human orthologs on each linkage group across all human chromosomes. If the observed number was more than three times the expected number (given an even distribution along a chromosome), we took this as evidence of an ancestral syntenic association of one or more human chromosomes. This procedure was repeated for comparisons between all other species. These data are presented in Supplementary Tables 1-6.

Results

Comparative Genomic Hybridization

We used comparative genomic hybridization (CGH) to reveal the sex specific region of the W chromosome in a female of representative python, colubrid and elapid snakes (Fig 1d–f). In female *L. fuscus*, we observed no sex specific signal on any chromosome. In *Dendrelaphis punctulatus* (Colubridae), the sex chromosomes are submetacentric and the fourth largest pair (Mengden 1982). CGH clearly identified the W chromosome as heavily biased with female-specific sequences along its length, though the distal long arm consistently produced a more intense signal (Fig 1e). In *Notechis scutatus*, the sex chromosomes are the fourth largest pair and the W is large and acrocentric (Shine & Bull 1977; Mengden 1981). Female-specific

sequences are found along most of the length of the W chromosome. The distal long arm of the W stains more intensely with DAPI and is not marked by the CGH probe (Fig 1f).

Chicken W chromosome paint and Bkm sequences

To investigate the extent to which bird sex chromosome repeats are amplified on the sex chromosomes of snakes, we used chicken W chromosome paint on representative snake species (Fig 1a–c). We also used *Bkm*-derived probes to examine the accumulation of this simple repeat on snake sex chromosomes (Fig 2a–f). FISH mapping of both simple and complex probes show patterns of repeat amplification concordant with the size of the female-specific region. In *L. fuscus*, no chromosomes were marked by these probes, indicating that neither the sequences found on the chicken W chromosome nor the *Bkm* repeat are amplified in this python. In *D. punctulatus* the distal region of the long arm showed noticeable accumulation of both classes of sequences, however the hybridization pattern and signal intensity differ between probes. In *N. scutatus*, the W chromosome shows heavy accumulation of *Bkm* related sequences along the length of the region identified as female-specific by CGH. The chicken W chromosome paint also marked this region but the signal was punctate and less intense. In the chicken, the W chromosome paint hybridized to all but the subtelomeric region of the short arm of the W, however no signal was observed with either *Bkm* probe (data not shown).

***18S rDNA and intronic sequences on the W chromosome of* Notechis scutatus**

We hybridized an 18S rDNA probe from the tammar wallaby to the metaphase chromosomes of male and female *N. scutatus* (Fig 3d,e). A single active NOR on chromosome 1 has previously been identified by silver staining (Mengden 1981). 18S rDNA maps to the same secondary constriction in both males and females. In females, however, the W chromosome shows massive amplification of 18S rDNA sequences along the length of the female-specific region. Hybridization of the two PCR-derived intron probes shows similar amplification on the W. The *DMRT1* intron 1 probe does not mark chromosome 2, where this gene is expected to map; instead, it shows an intense signal along the female-specific region of the W (Fig 3a). The *CTNNB1* terminal intron probe shows a similar amplification on the W, but no signal on the Z chromosome, where the gene is located in all snakes so far examined (Fig 3c). The short arm of chromosome 6 has a large distal heterochromatic region (Mengden 1982) that is also marked by this probe. The phage-derived DMRT-related probe marked subtelomeric regions on Zp and Wq (Fig 3b). Hybridization on the W chromosome corresponds with the terminal DAPI-bright heterochromatic region. Again, we observed no signal on chromosome 2. PCR confirmed the presence of the conserved intronic region "A" in the probe (Brunner et al., 2001; data not shown) and Stiglec (2007) amplified sequences from exons 3 and 4. We use the term 'DMRT-

related' for the repeats identified with this probe. None of the intronic probes described hybridized to chicken metaphase chromosomes (data not shown).

We sequenced the cloned 5.4kb DMRT1 and 1.2kb CTNNB1 introns using a primer walking strategy in an attempt to characterize the repetitive elements contained therein (Genbank accession numbers HM559261 and HM559262 respectively). Dotplots reveal no significant tandem duplications (not shown). Searching for repeats using RepeatMasker (http://repeatmasker.org) failed to identify any repetitive element in either intron; however, *DMRT1* intron 1 contains some extended mononucleotide stretches. We searched the GenBank non-redundant (nr) database by restricting the query to Serpentes (snakes). *DMRT1* intron 1 revealed homology to intronic sequences of *MYH2* and a number of venom genes (85-96% identity over 45-134bp) and flanking sequences of microsatellites. No such pattern was observed for the terminal intron of *CTNNB1*, however, when querying all of the non-redundant database, short regions of homology were found with chicken BAC clone CH261-55O13 (82% identity over 45 bases) and human BAC clone RP11-281O15 (70% identity over 75 bases). These clones map to the chicken Zp (20.72Mb) and human 5q35.3, respectively (UCSC Genome browser http://genome.ucsc.edu).

Analyses of ancestral syntenies

We investigated the possibility of an ancestral association of chicken (GGA) and snake Z chromosomes by examining the location of orthologs in humans (HSA), *Xenopus tropicalis* (XTR) and zebrafish (DRE) (see Supplementary Tables 1-6). First, we looked for ancestral synteny of orthologs from GGA2 and GGA27 to determine the antiquity of the snake Z chromosome. GGA2 shares regions of orthology with HSA7, 8, 18 and Y and GGA27 is equivalent to HSA17; in *Xenopus*, GGA2=XTR2 and GGA27=XTR10; in zebrafish, GGA2=DRE2, 16, 19 and 24 and GGA27=DRE3, 11 and 12. In each species, none of the chromosomes orthologous to GGA2 is common with those orthologous to GGA27, suggesting that the snake Z arose more recently than did the tetrapod or amniote ancestor. To look for ancestral synteny of chicken and snake Z chromosomes, we examined the location of GGAZ orthologs in humans, *Xenopus* and zebrafish and compared their locations to GGA2 and GGA27 orthologs, as already identified. In humans, GGAZ=HSA5, 9, 18; in *Xenopus*, GGAZ=XTR2; in zebrafish, GGAZ=DRE5, 8, 10, 21. Genes on the snake Z (GGA2) and GGAZ are syntenic in HSA18 only, but synteny of these genes in a tetrapod ancestor is not supported.

Discussion

Repeat accumulation on snake W chromosomes

We mapped the distribution of a number of repetitive DNA sequences on the W chromosomes of snakes whose W chromosomes differ in the degree of differentiation. The pattern of repeat accumulation shows that W chromosome differentiation is accompanied by an increasingly complex suite of repetitive DNA, the distribution of which coincides with the female-specific region of the W chromosome. In the water python, *L. fuscus*, we found no evidence of any amplified sequences on the W chromosome (Figs 1a, d & 2a, d). This is consistent with previous G and C banding studies that identified no cytological differences between the chromosomes of males and females in this species (Mengden & Stock 1980; Mengden 1982). CGH should be sensitive enough to detect differential segments as small as 2-3Mb (Schoumans et al. 2004). Our failure in identifying the female-specific region of the python W may be due to differential amplification of repeats common to both sexes. It could also be explained by the use of male DNA from the congener *L. olivaceous*; however, we should still have identified female-specific regions using this probe, if present. Sex chromosomes in both species are yet to be demonstrated cytologically, but are presumed to be the fourth largest pair, as in many other snakes (Mengden & Stock 1980).

In the colubrid and elapid species included in this study, differentiated sex chromosomes have previously been characterized by standard cytogenetic techniques (Shine & Bull 1977; Mengden 1981, 1982). In *D. punctulatus* the W chromosome differs from the Z in centromere position (the short arm is slightly shorter), and C banding reveals it to be largely heterochromatic (Mengden 1982). We show that female-specific sequences are found along its length, but concentrated on the distal region of the long arm (Fig 1e). This same distal region is heavily marked by *Bkm* probes and the chicken W chromosome paint. In *N. scutatus*, a similar pattern is observed; however, the majority of the W chromosome bears female-specific sequences, *Bkm*, *DMRT1*-releated, *CTNNB1*-related and 18S-related repeats and chicken W chromosome sequences. A different hybridization pattern for each probe suggests that each class of repeats is unique.

Bkm is often, but not always associated with heterochromatin both in autosomes and degenerated sex chromosomes (Jones & Singh 1985; Nanda et al. 1991). Our data are in agreement with previous studies in which Southern hybridization was used to demonstrate a positive relationship with the degree of *Bkm* accumulation and W chromosome degeneration (Jones & Singh 1985). While a common origin of *Bkm* repeats has been assumed (e.g. Epplen et al. 1983), the simple sequence and tandem structure is prone to amplification by slipped-

strand mispairing and similar replicative errors, so independent origins are equally likely (Epplen 1988). Proteins that specifically bind *Bkm* have been isolated from the gonads of snakes, silkworms, mice and man (Singh et al. 1994; Priyadarshini et al. 2003). A role in regulating the decondensation and transcriptional activation of Y or W heterochromatin has been suggested but remains to be demonstrated (Singh et al. 1994). The absence of this repeat in large domains on the sex chromosomes of pythons argues against a conserved, functional role for *Bkm*.

Searching RepeatMasker with sequence from the first intron of *DMRT1* and the terminal intron of *CTNNB1* did not reveal any known repetitive element. Sequence similarity with introns of other, unrelated genes suggests that they may contain extinct and now degenerate transposable elements. We suggest that the hybridization signal occurs because the probes recognize transposable elements that have been greatly amplified on the degenerated W chromosome of *N. scutatus*. In addition to a W chromosome signal, the *CTNNB1*-related repeat is amplified in a region of heterochromatin on chromosome 6 (Fig 3c). The DMRT-related phage clone contains repeats common to both the Z and W chromosome, though the size of the domain occupied by these repeats is larger on the W chromosome (Fig 3b). This explains why the subtelomeric region of the W chromosome long arm was unmarked by the CGH probe (Fig 1f). Its distribution is coincident with a heterochromatic region identified by C banding (Mengden 1982). The co-location of heterochromatin and putative transposable elements supports a recent suggestion that heterochromatin accumulates as a cellular defense against unchecked activity of retrotransposons (Steinemann & Steinemann 2005).

The nucleolus organizer region (NOR) is located on chromosome 1 of *N. scutatus*, as for most elapid snakes (Mengden 1982). Silver staining shows that this is the only active NOR, at least in somatic tissues (Mengden 1981). We confirmed its autosomal location using FISH but found that 18S-related repeats are also amplified on the W chromosome (Fig 3d-e). This is also the case for the W chromosomes of the colubrids *E. quadrivirgata* and *Boiga irregularis* (O'Meally, unpub data). In the crotalid viper *Agkistrodon contortrix*, active NORs have been mapped to the Z chromosome, but a female was not examined (Porter et al. 1991). In the turtle *Pelodiscus sinensis*, active NORs have been mapped to both sex chromosomes (Kawai et al. 2007). In females of this species, the W chromosome is marked almost entirely by an 18S+28S rDNA FISH probe, and silver staining shows that ribosomal proteins accumulate in a ladder-like fashion along its length. We suggest that the repetitive nature of the NOR lends itself to amplification on the sex chromosomes of these species. There may also be functional significance for sex-linked NORs. During oogenesis in some amphibians, transcription of ribosomal DNA is elevated to produce the large number of ribosomes required for early embryo development (Gall 1968;

Roger et al. 2002). To our knowledge, no investigation of the activity of NORs has been made during reptile oogenesis, but W-specific amplification of this locus may facilitate elevated ribosome production. It would be interesting to investigate the activity of W-linked ribosomal sequences during oogenesis in these species.

Evolution of snake sex chromosomes

Comparative mapping of sex-linked genes in three species of snakes indicates that W chromosome differentiation has proceeded from the short arm (Matsubara et al. 2006). The conserved *Bam*HI repeat, isolated from *E. quadrivirgata*, maps to the short arm of the Z and W in *Python molurus* and *Protobothrops flavoviridis.* In *E. quadrivirgata*, this repeat is found on the short arm of the W but the long arm of the Z chromosome, suggesting the centromere has been repositioned. The order of gene loss on the degenerated W chromosome of *E. quadrivirgata* and *Protobothrops flavoviridis* extends from this conserved repeat (Matsubara et al. 2006). In the elapid Pseudonaja textilis, the short arm of the W also bears the greatest density of repeats (*Chapter 2*). Our mapping in the colubrid *D. punctulata* suggests the centromere has been repositioned, as repeats and heterochromatin are located on the long arm of the W chromosome. In the elapid *N. scutatus*, DMRT-related repeats map to the short arm of the Z and the long arm of the W. This implies that the W chromosome has undergone considerable rearrangement, including centromere repositioning and expansion of repetitive content. The large size of the W chromosome may indicate a recent addition of repetitive DNA following rearrangement, as this is thought to occur shortly after recombination is suppressed (Parker 1990; Charlesworth et al. 2005). In a wide survey of elapid karyology, Mengden (1982) noted that the W chromosome is the most variable element in the snake genome, even between closely related taxa. This suggests that W chromosome differentiation occurs repeatedly after speciation, and contrasts with the gradual accumulation of differences suggested for some other taxa (Lahn & Page 1999; Nicolas et al. 2005).

While the W chromosome is highly variable, the snake Z chromosome is remarkably well conserved, even between snakes and some agamid lizards. In *Pogona vitticeps,* six genes that map to chromosome 6p are located on Zp in *E. quadrivirgata* and chicken 2p (Ezaz et al. 2009b). On chromosome 6 in *Leiolepis reevesii reevesii*, synteny of four snake Z genes is conserved; one maps to GGA27 and three to GGA2 (Srikulnath et al. 2009). Because these genes are autosomal in agamid lizards, a sex-determining role for this ancestral chromosome likely came about after early snakes diverged from other Toxicofera, but before pythons arose (105MYA). The snake Z could have operated as a sex chromosome in a common ancestor but lost that role in agamids, although wider taxonomic sampling is required to test this. In any case, synteny of these genes has been conserved since the divergence of snakes and agamids

from a common ancestor, about 166 million years ago (Hedges et al. 2006). In all other amniotes and tetrapod outgroups, genes from GGA2 and 27 do not share synteny, suggesting that the snake Z originated later than did a common amniote ancestor. Interestingly, it appears that GGA2 and 27 derive from the same ancestral vertebrate chromosome 'E' suggested by Nakatani et al. (2007), prior to two rounds of genome duplication. This situation is analogous to the conserved and added regions of the eutherian X chromosome (Graves 1998; Glas et al. 1999), which in large part derive from the ancestral vertebrate chromosome 'F' (Nakatani et al. 2007). Perhaps this ancestral homology predisposed their subsequent fusion in ancestral amniotes.

Implications of sex-chromosome repeats shared between snakes and birds

Hybridization of chicken W chromosome paint to the W chromosome of derived snakes suggests sequences are shared, despite 275 million years of independent evolution. This could be explained by ancestral synteny of amniote sex chromosomes, as recently suggested by Smith & Voss (2007). We examined the location of orthologs from GGAZ and those from GGA2 and 27 (as a proxy for snake Z genes) in amniotes and tetrapod outgroups, but found no evidence of large-scale synteny conservation. Of the 236 chicken-human orthologs on HSA18, 176 map to GGA2 (22 would be expected given an even distribution over all chromosomes) and 48 map to GGAZ (11 expected); 12 are found on other chicken chromosomes. None of the 176 GGA2 orthologs has yet been mapped in snakes, so this should not be considered strong evidence for an ancestral association of chicken and snake Z chromosomes. The synteny of HSA18 genes may have arisen independently in the mammalian lineage and so represent a derived rather than an ancestral state; this scenario is implied by the ancestral amniote karyotype suggested by Nakatani et al. (2007). In tuatara, orthologs from snake, bird and mammalian sex chromosomes map to different autosomes (although the map coverage is light), as is the case in *Xenopus* (Chapter 4: O'Meally et al. 2010; Supplementary Tables).

Another possibility is that these repeats have arisen on snake and chicken W chromosomes by convergence. The chicken W chromosome is largely composed of three repeat families, named for the restriction enzymes with which they were isolated. The *Xho*I and *Eco*RI families consist of 0.6-0.7kb unit containing 21bp tandem repeats organized around different W chromomeres; together they account for 65% of W chromosome DNA and are thought o be involved in heterochromatin assembly (Tone et al. 1984; Kodama et al. 1987; Saitoh & Mizuno 1992; Suka et al. 1993; Solovei et al. 1998). The *Ssp*I family consists of a 0.5kb unit with a terminal 120 bp tandem repeat that is GA rich, and accounts for about 10% of W chromosome DNA (Itoh & Mizuno 2002). Southern blot experiments across Galliformes and Neoaves show that these three repeat families are restricted to the genus *Gallus* and absent from the genomes of other

birds (Tone et al. 1984; Itoh & Mizuno 2002). This suggests that no more than 20-25% (about 10Mb) of the *Gallus* W is homologous with other birds, and that the snake W repeat must have an analog in this sequence. We attempted to replicate the W paint hybridization pattern using the largest (CH261-114G22; 167.4kb) mapped chicken W BAC clone from the sequenced euchromatic region, but without success. Southern hybridizations of chicken W paint on male and female genomic DNA from snakes also failed to produce a female specific band that could be isolated. Without having characterized the repeat by sequence analysis, it is difficult to speculate on its origins and possible functional roles. In the absence of ancestral sex chromosome synteny, we suggest the repetitive content must be due to convergent degeneration. Given the large evolutionary distance between birds and snakes, this is likely a general property of sex chromosome differentiation.

Acknowledgements

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) under permits and licenses issued by Environment ACT, the New South Wales State and Northern Territory governments (LI2008321, S12364 & 26791 respectively) and with the approval of the Australian National University Animal Experimentation Ethics Committee (Proposal R.CG.07.3b/04/07). We are grateful to John Cann and Paul Waters for providing specimens or assisting with collection.

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Tables and Figures

Table 1. Material examined. Collection localities are within Australia. [a] The male examined was from the closely related congener *Liasis olivaceus* (ID# Lo0308-4). [b] These samples derive from a sibling pair, hatched from the same egg clutch.

Familly	Species	Number of individuals examined (F/M)	Collection locality
Pythonidae	*Liasis fuscus*	2/1[a]	Daly River, NT
Colubridae	*Dendrelaphis punctulatus*	1/1[b]	Fogg Dam, NT
Elapidae	*Notechis scutatus*	4/3	Goulburn, NSW

Table 2. Oligonucleotide primers used for PCR and sequencing of *DMRT1* intron 1 and the terminal intron of *CTNNB1*. See text for product sizes and reaction conditions. Sequencing primers are indicated by a numerical suffix.

Primer name	Sequence (5'→3')
DMI1F	AGCAGGCAGGAACTGTTAGCGTTG
DMI1R	TGCATGTGGAGAGATTGCCAGTGT
DMI1F-1	CGATAACTAAAGGGAAAAACAAGTC
DMI1F-2	GGTTTGTGCTGGTTTGCTTT
DMI1F-3	TCCCAGAATTGAGCTAACATGA
DMI1F-100	TTGCTTACATTGCAACAGCA
DMI1F-101	GGGATGATGGGTGTTGAAGT
DMI1R-1	GATCCCAGCTCTTCCTCTT
DMI1R-2	AATGCTTTTCCAATGCAACG
DMI1R-3	CAATCAACTATTGTATGGGAGCA
DMI1R-4	TCCTAACCTTGCTGGAGTGAC
DMI1R-100	CAGGCAGATCTTTCCTGAGC
DMI1R-101	TGCAGACTCTGATGCCTTTG
CTNNB1F	ACTGAACCAATGGCTTGGAA
CTNNB1R	ACCAGTTGCCTTTTATCCCA
CTNNB1F-3	GTGGCAAAGGAAATCCTGAA
CTNNB1R-1	CTCTGCCCAGCAAATCATG

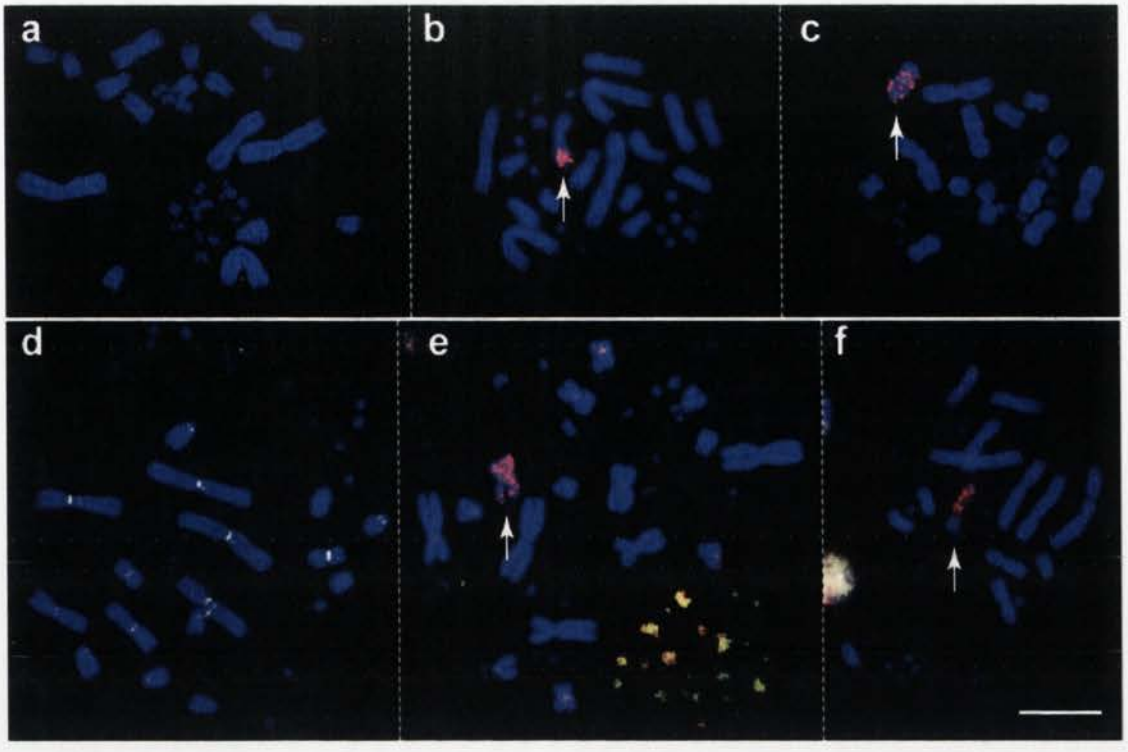

Figure 1. Hybridization of chicken W chromosome paint (upper images) and comparative genomic hybridization (lower images) of male (green) and female (red) genomic DNA to female metaphase spreads of (**a,d**) *Liasis fuscus*, (**b,e**) *Dendrelaphis punctulata* and (**c,f**) *Notechis scutatus*. Arrows indicate the W chromosome and the scale bar represents 10μm.

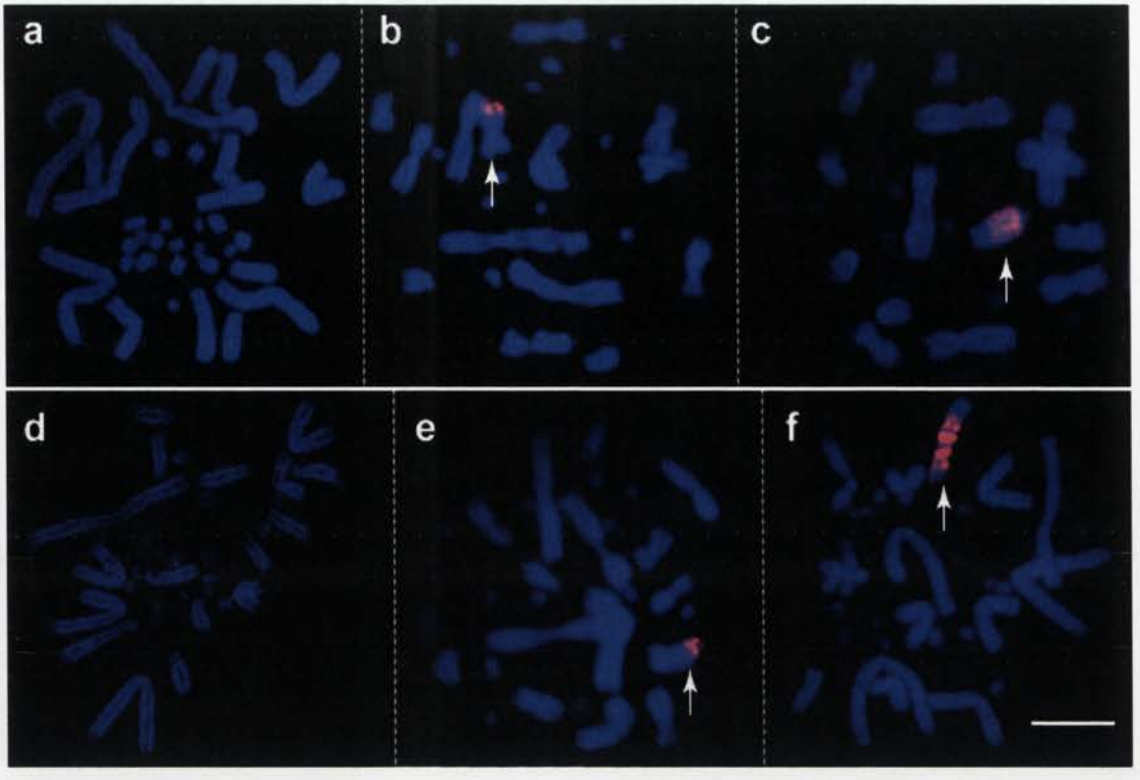

Figure 2. Hybridization of *Bkm*-related oligonucleotide probes to female metaphase spreads of (**a,d**) *Liasis fuscus*, (**b,e**) *Dendrelaphis punctulata* and (**c,f**) *Notechis scutatus*. The probe is $(GATA)_4$ in upper images and $(GACA)_7$ in lower images. Arrows indicate the W chromosome and the scale bar represents 10μm.

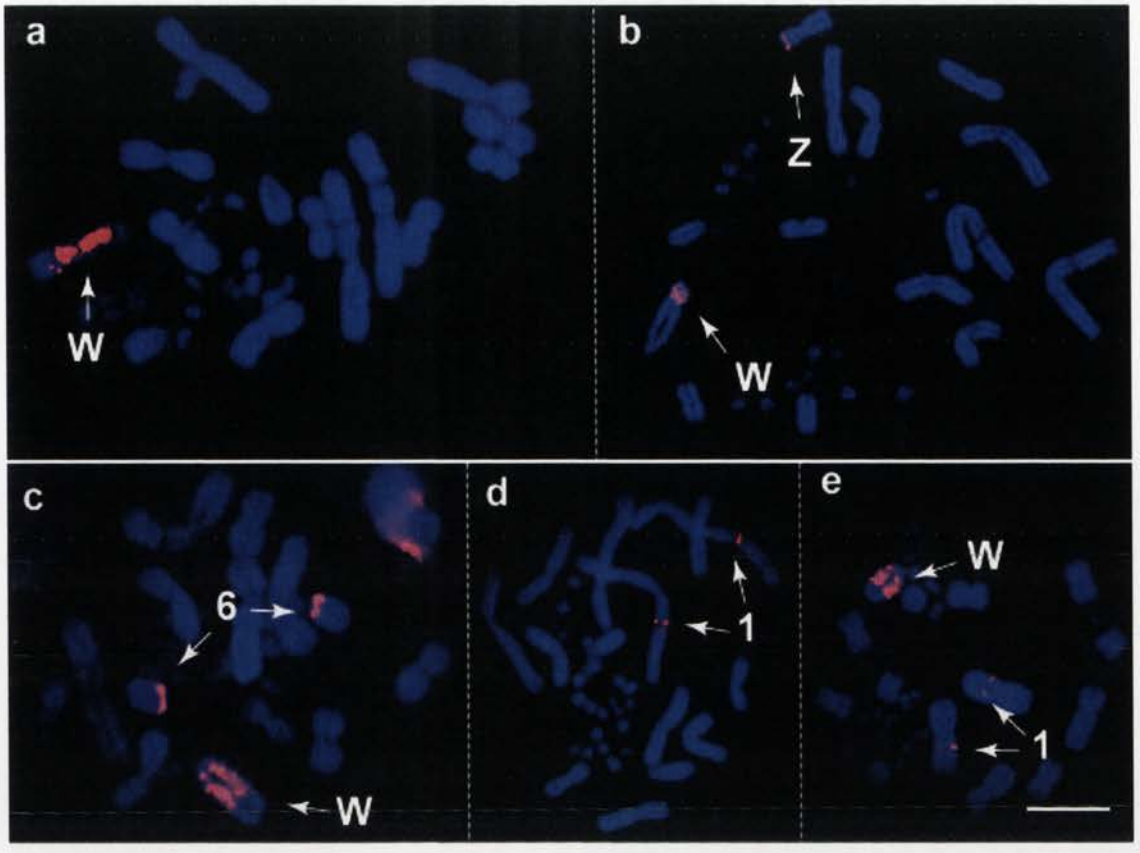

Figure 3. Hybridization of homologous probes containing (**a**) intron 1 of *DMRT1*, and the terminal introns of (**b**) *DMRT1*, and (**c**) *CTNNB1* from *Notechis scutatus* on female metaphase chromosomes. (**d,e**) Heterologous 18S rDNA probe hybridized to *N. scutatus* male and female metaphase chromosomes respectively. The scale bar represents 10μm.

Supplementary Tables

Supplementary Tables S1-S6. Oxford grids showing the number of orthologs that map to the chromosomes (or linkage group) of one species (columns) and those of another species (rows). Table S1 shows comparisons between human (HSA) and chicken (GGA), Table S2 between human and *Xenopus* (XTR), Table S3 between human and zebrafish (DRE). Table S4 shows comparisons between chicken and *Xenopus*, Table S5 between chicken and zebrafish; and table S6 between *Xenopus* and zebrafish. Given an even distribution along a chromosome (or linkage group), the expected number of genes (*E*) for each cell can be calculated as the product of the row total and column total, divided by the total number of orthologs for the table (given at the lower right of each table). Cells are colored to indicate the extent to which the observed number of genes (*O*) deviates from the expected value: blue where $O < E$ indicating no ancestral association; green $O < 2 \times E$ indicating a weak association; yellow $O < 3 \times E$ indicating an intermediate association; and red $O \geq 3 \times E$ indicating a strong ancestral association. Where a chromosome from one species (columns) has intermediate or strong associations with more than one chromosome from the other species (rows) in a table, ancestral synteny of these genes can be inferred. For example, in Table S1, the chicken Z (695 human orthologs) shows strong ancestral associations with human 5 (317 orthologs); human 9 (276 orthologs) and human 18 (48 orthologs); 54 orthologs of chicken Z genes are distributed on other human chromosomes, but no more than would be expected given an even distribution along all chromosomes. Further support for such associations can be gained by examining the corresponding regions in *Xenopus* and zebrafish.

Note that in tables comparing chicken (S1, 4 & 5), chromosomes 29 – 32 are omitted as no genes have been mapped to these chromosomes in the current (WASHU 2.1, May 2006) chicken assembly. In Table S2, the human Y is omitted as no *Xenopus*-human Y orthologs have been mapped in *Xenopus*. In Table S4, the chicken W is omitted as no *Xenopus*-chicken W orthologs have been mapped in *Xenopus*.

Table S1. Human (HSA) vs chicken (GGA) Oxford grid. See page 67 for explanatory notes.

GGA\HSA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Totals
1	79	65	129	8	6	57	215	6	16	45	218	441	278	4	22	7	11	2	4	2	127	210	182	13	2147
2	13	2	183	6	79	99	302	329	32	97	2	7	6	3	6	6	1	176	17	10	0	3	2	14	1395
3	172	310	0	8	5	518	2	53	1	1	5	1	0	1	2	2	4	0	2	74	1	0	1	0	1163
4	1	40	1	630	6	5	0	44	4	2	5	3	6	15	3	1	4	1	4	18	0	1	280	12	1086
5	2	2	4	7	1	4	3	1	6	0	404	0	1	395	84	0	22	1	5	0	0	0	4	0	946
6	1	4	0	10	0	0	1	2	3	522	1	2	0	9	3	1	0	0	0	1	0	1	3	0	564
7	2	410	36	0	1	0	3	1	0	12	2	2	0	1	0	3	1	0	8	1	16	0	1	0	500
8	472	0	0	1	4	2	1	0	4	1	1	2	0	8	2	4	0	0	4	1	0	0	1	0	508
9	0	120	287	0	0	0	6	0	0	0	2	0	4	0	0	0	0	0	1	1	9	0	1	0	431
10	2	5	3	1	2	3	6	2	0	0	5	2	0	0	397	1	2	0	1	0	0	2	1	0	435
11	0	0	3	3	3	1	2	8	0	0	0	2	0	8	1	306	0	0	36	0	0	0	1	1	375
12	2	3	272	0	1	0	0	0	20	0	0	0	0	0	0	1	0	0	1	0	0	0	5	0	305
13	0	1	0	0	317	2	0	1	0	0	2	0	0	0	2	0	0	0	0	0	0	0	1	0	326
14	0	1	5	6	1	0	100	8	1	3	5	2	0	1	0	276	37	1	3	0	1	0	3	1	455
15	0	2	0	0	1	1	0	0	0	0	0	194	0	1	0	0	0	0	1	1	0	172	1	0	374
16	9	0	0	0	13	13	0	0	0	0	0	6	0	1	0	0	0	0	2	0	0	0	0	0	44
17	1	4	1	1	1	8	0	1	273	1	0	0	1	0	60	1	0	1	2	0	0	1	1	0	358
18	3	0	0	0	0	0	1	0	0	0	0	2	0	0	0	0	310	0	2	0	0	4	3	0	325
19	0	0	0	0	16	6	92	0	3	0	4	0	1	1	0	4	232	2	0	2	0	4	1	0	368
20	0	0	0	4	0	0	0	4	1	0	0	0	1	0	4	0	0	0	3	317	0	0	0	0	334
21	236	0	1	1	0	0	1	0	0	1	1	0	0	0	0	3	1	0	1	0	0	0	0	0	246
22	0	26	1	0	0	0	4	79	0	4	0	0	0	0	0	0	0	0	0	7	0	0	0	0	121
23	210	0	1	2	2	3	0	0	0	0	0	0	0	4	0	0	1	4	0	0	0	0	2	0	229
24	0	0	0	0	0	0	0	0	0	0	153	0	1	0	0	0	0	0	2	0	0	0	1	0	157
25	88	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	92
26	156	0	0	0	1	69	0	4	1	0	0	0	0	0	0	2	0	0	0	0	0	1	2	0	236
27	0	2	0	1	1	1	2	0	1	0	1	1	1	2	3	0	191	0	0	0	0	0	1	0	208
28	1	2	0	2	2	0	2	20	0	0	1	0	0	0	0	1	3	0	185	0	0	0	0	0	219
Z	6	8	2	10	317	2	2	10	276	0	2	0	0	1	4	2	0	48	1	1	0	0	3	0	695
W	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Totals	1456	1007	930	701	780	794	745	573	643	690	814	667	300	455	593	621	820	236	285	438	154	399	501	41	∑14643

Table S2. Human (HSA) vs *Xenopus* (XTR) Oxford grid. See page 67 for explanatory notes.

XTR\HSA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Totals
LG1	21	50	9	312	159	5	9	63	218	9	21	98	5	79	23	13	17	44	113	25	18	132	23	1466
LG2	14	4	164	5	46	75	202	191	11	74	3	4	4	1	4	4	3	65	14	1	1	5	5	900
LG3	23	225	32	2	3	21	51	3	5	15	12	4	1	2	14	189	51	1	2	1	20	2	3	682
LG4	108	3	27	2	1	2	5	4	10	198	138	29	2	0	9	6	1	0	115	1	1	1	4	667
LG5	28	12	3	9	12	51	10	4	92	10	12	4	4	272	113	7	8	2	89	1	1	2	162	908
LG6	135	43	53	9	20	7	39	9	4	1	89	134	108	4	5	4	63	6	22	6	53	7	39	860
LG7	191	1	110	10	4	9	15	2	8	1	260	14	2	1	6	143	16	2	11	1	0	65	5	877
LG8	4	13	2	2	187	7	40	5	13	2	4	135	0	4	109	3	4	5	1	1	1	47	4	593
LG9	117	135	24	8	3	230	3	9	3	0	9	3	1	3	3	2	0	1	2	30	2	1	6	595
LG10	4	0	1	0	3	2	0	0	0	1	3	0	0	1	5	4	213	0	3	49	0	1	3	293
Totals	645	486	425	359	438	409	374	290	364	311	551	425	127	367	291	375	376	126	372	116	97	263	254	∑7841

Table S3. Human (HSA) vs zebrafish (DRE) Oxford grid. See page 67 for explanatory notes.

DRE\HSA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Totals
1	47	75	32	250	3	46	62	22	52	30	49	6	78	2	5	48	45	4	81	16	28	30	29	5	1045
2	248	46	136	2	27	26	60	62	9	52	5	9	1	42	4	4	9	36	108	2	1	7	6	4	906
3	20	18	10	19	16	67	147	14	37	5	50	23	2	10	6	242	321	2	150	2	1	76	4	0	1242
4	24	11	26	10	2	52	117	4	13	57	89	233	0	4	7	4	4	0	8	4	0	53	8	0	730
5	18	49	16	42	171	18	46	28	210	2	122	104	10	13	53	7	105	11	35	8	0	78	73	0	1219
6	161	155	156	6	4	25	7	10	11	16	3	87	23	1	19	12	43	2	23	86	7	30	19	1	907
7	28	20	8	52	7	74	50	29	30	9	207	8	1	40	162	154	53	9	50	10	1	40	25	2	1069
8	203	35	56	11	53	130	21	78	102	12	23	67	1	5	2	12	5	9	53	43	1	52	80	15	1069
9	40	447	75	2	9	1	5	12	0	7	11	17	106	4	2	3	3	0	4	1	50	2	47	5	853
10	16	26	13	18	188	61	43	37	74	5	82	31	38	76	13	2	56	10	13	5	38	54	15	0	914
11	146	32	105	5	6	23	7	3	15	4	8	29	3	5	2	9	40	2	64	49	5	0	42	1	605
12	14	35	7	15	16	11	39	6	12	191	17	2	0	6	3	65	211	2	26	1	0	26	4	0	709
13	90	111	9	42	3	113	9	18	7	254	18	5	0	105	6	3	1	1	15	42	1	4	6	0	863
14	9	17	6	114	461	11	12	21	13	7	80	6	4	9	3	3	11	3	11	6	0	3	139	9	958
15	31	30	71	4	6	14	56	14	5	14	154	7	28	4	7	9	148	1	115	1	17	8	1	0	745
16	189	3	99	2	13	88	130	101	15	9	11	42	4	8	1	5	9	26	152	4	2	3	4	0	920
17	106	101	1	12	3	91	9	20	0	78	5	2	5	214	48	1	7	2	5	20	0	1	7	0	738
18	7	11	30	2	11	10	32	6	3	5	111	53	1	3	125	110	2	8	59	0	0	22	5	0	616
19	197	9	38	8	44	81	103	105	7	8	6	17	2	3	5	13	39	30	72	5	0	19	5	4	820
20	166	103	6	45	3	250	16	38	6	3	7	10	0	138	25	9	27	26	7	16	1	2	4	0	908
21	19	9	10	35	197	4	25	13	101	1	100	26	6	1	3	2	53	27	12	5	3	19	53	2	726
22	96	43	128	2	5	25	14	12	12	13	39	25	0	12	4	16	28	6	139	9	6	11	7	0	652
23	182	23	45	26	6	45	13	9	14	0	6	129	1	6	2	4	38	3	7	133	0	5	63	0	760
24	20	24	104	5	14	24	50	65	21	49	4	2	23	20	3	38	18	39	19	2	7	19	25	2	597
25	4	21	4	2	10	158	78	4	13	3	127	69	0	3	155	74	33	4	19	0	1	22	14	0	818
Totals	**2081**	**1454**	**1191**	**731**	**1278**	**1448**	**1151**	**731**	**782**	**834**	**1334**	**1009**	**337**	**734**	**665**	**849**	**1309**	**263**	**1247**	**470**	**170**	**586**	**685**	**50**	**∑21389**

Table S4. Chicken (GGA) vs *Xenopus* (XTR) Oxford grid. See page 67 for explanatory notes.

XTR\GGA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Z	Totals
LG1	11	12	1	369	5	3	4	7	7	8	4	0	10	3	202	1	1	0	10	2	0	1	0	0	6	1	14	62	372	1116
LG2	18	750	4	0	5	0	3	1	37	0	2	0	3	2	2	0	1	0	0	0	0	0	2	0	2	0	2	8	0	842
LG3	17	5	13	0	1	0	222	4	28	13	0	0	4	211	0	0	2	6	18	1	0	0	8	1	0	7	1	0	2	564
LG4	33	3	5	1	5	188	1	5	22	2	2	1	0	2	5	1	0	2	0	1	89	1	1	105	5	1	1	0	4	486
LG5	7	1	15	142	323	8	4	5	2	2	1	1	3	0	0	19	84	0	0	0	1	0	0	0	2	0	1	2	2	625
LG6	347	7	0	4	1	0	9	0	2	3	0	1	3	0	1	0	1	1	79	4	1	0	108	1	1	0	2	0	1	577
LG7	82	6	12	11	187	1	0	153	1	1	138	93	0	2	2	0	2	0	0	0	0	0	0	1	5	0	1	2	4	704
LG8	221	0	8	0	2	1	2	0	0	90	0	0	163	0	0	0	0	1	2	1	0	0	0	0	0	1	0	0	3	495
LG9	5	1	472	2	8	0	1	2	30	0	1	3	0	0	0	0	2	0	1	2	2	0	1	1	0	1	0	0	0	535
LG10	2	3	2	0	2	0	0	1	0	1	1	0	4	0	0	1	0	79	2	52	0	0	0	0	0	0	64	0	0	214
Totals	743	788	532	529	539	201	246	178	129	120	149	99	190	220	212	22	93	89	112	63	93	2	120	109	21	11	86	74	388	∑6158

Table S5. Chicken (GGA) vs zebrafish (DRE) Oxford grid. See page 67 for explanatory notes.

DRE\GGA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Z	W	Totals
1	194	74	35	253	24	31	36	7	4	3	0	1	3	67	7	39	0	16	3	0	0	0	2	1	0	0	0	0	45	0	845
2	15	272	8	3	2	10	4	179	132	4	2	4	1	3	3	1	3	1	2	0	0	0	4	1	2	0	3	62	3	0	724
3	122	143	43	5	9	0	5	5	1	4	7	0	1	256	7	69	2	129	8	2	1	0	3	0	0	1	115	1	2	0	941
4	441	7	22	3	19	1	0	1	0	1	1	8	0	0	0	1	0	0	0	0	0	1	2	13	1	0	0	1	12	0	535
5	41	22	7	112	19	3	5	2	6	2	0	1	2	2	153	1	132	7	75	0	2	20	6	41	0	1	1	3	206	0	872
6	114	15	16	2	1	4	116	93	47	2	1	92	0	8	12	0	7	37	0	82	14	1	1	0	0	46	2	0	5	0	718
7	24	82	7	83	158	2	1	5	12	140	153	0	1	4	4	3	0	0	3	2	1	0	1	0	3	0	1	1	26	0	717
8	18	8	6	23	4	5	1	67	0	1	4	41	1	0	98	39	49	3	1	27	52	54	0	0	0	59	0	21	89	1	672
9	283	14	1	9	4	3	293	6	15	2	1	2	6	0	11	1	0	0	0	1	1	2	0	0	2	0	0	0	5	0	662
10	117	9	20	31	3	2	1	0	1	1	1	0	46	1	60	5	21	1	45	0	0	39	0	27	1	0	1	0	122	0	555
11	29	6	33	3	6	1	23	18	21	0	4	102	2	3	3	1	3	6	3	48	46	2	1	1	0	55	28	34	2	0	484
12	32	29	37	7	3	161	4	2	0	3	0	0	7	63	2	1	2	109	0	1	0	0	0	1	1	0	38	0	6	0	509
13	22	16	272	45	88	243	2	2	2	0	1	0	5	1	2	0	3	2	0	0	0	0	31	0	0	0	0	0	3	0	740
14	10	4	11	290	10	5	2	0	0	1	1	0	245	0	6	1	0	0	2	0	0	0	0	0	0	0	1	5	12	0	606
15	113	30	10	7	9	9	0	1	81	0	1	0	1	2	2	2	1	1	131	0	0	1	1	55	0	0	3	0	3	0	464
16	63	391	44	1	3	1	4	7	0	0	1	0	0	4	0	3	2	2	6	2	1	0	46	7	43	2	1	2	6	0	642
17	4	10	243	14	245	69	0	10	0	10	0	2	1	0	2	0	0	5	5	2	0	1	32	1	0	0	2	1	3	0	662
18	123	7	1	0	59	0	0	3	31	111	120	1	1	0	0	5	0	0	1	0	1	0	0	20	0	1	0	0	4	0	489
19	18	285	22	0	0	2	6	1	0	1	2	0	0	1	2	23	5	9	0	4	0	0	97	0	29	0	16	1	5	0	529
20	10	46	398	32	149	2	0	116	0	6	2	0	3	0	1	0	1	0	16	4	3	0	1	0	0	1	0	2	3	0	796
21	59	5	3	81	4	2	2	0	5	1	0	1	122	0	38	0	73	3	55	0	1	6	11	19	0	0	2	0	109	2	604
22	32	22	31	4	29	15	34	71	51	3	1	47	0	9	0	1	4	5	0	1	7	1	0	4	2	33	13	84	7	0	511
23	16	22	38	21	3	1	12	12	0	1	0	43	1	0	1	0	4	0	0	121	112	1	0	0	0	45	4	0	7	0	465
24	78	246	1	1	4	0	1	18	46	0	2	0	0	36	7	0	6	2	0	1	0	0	0	1	0	0	5	0	3	0	458
25	183	23	2	2	129	0	1	0	0	125	81	0	4	0	4	0	0	1	14	0	1	0	1	0	0	0	2	1	3	0	577
Totals	2161	1788	1311	1032	984	572	553	626	455	422	386	345	453	460	425	196	318	339	370	298	243	129	240	192	84	244	238	219	691	3	∑15777

Table S6. Xenopus (XTR) vs zebrafish (DRE) Oxford grid. See page 67 for explanatory notes.

XTR\DRE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Totals
LG1	220	107	74	64	232	17	86	169	53	130	16	19	36	102	13	24	13	4	18	25	138	79	21	21	11	1692
LG2	8	197	14	45	9	14	58	6	11	2	15	17	11	0	18	216	3	7	213	10	1	14	10	178	18	1095
LG3	80	10	165	19	15	84	25	11	169	12	23	70	4	3	79	12	6	7	16	16	167	42	17	31	16	1099
LG4	44	24	78	136	47	12	19	45	7	32	26	85	114	6	122	97	49	28	22	5	21	45	55	16	12	1147
LG5	12	10	16	14	173	3	26	56	11	12	11	5	67	74	51	15	198	30	23	117	72	4	28	3	2	1033
LG6	56	7	21	10	35	52	9	11	135	101	31	3	21	4	130	28	26	21	66	8	49	28	88	17	1	958
LG7	24	72	54	86	31	113	231	30	19	12	53	18	6	37	13	17	9	86	10	49	24	73	21	29	108	1225
LG8	4	3	3	149	7	3	63	4	5	5	4	8	2	112	0	0	5	60	1	6	81	5	2	1	109	642
LG9	13	14	15	18	9	12	10	5	1	1	25	31	154	4	9	36	108	8	6	159	1	27	28	2	12	708
LG10	3	5	105	0	8	32	4	10	3	0	24	60	1	0	2	2	4	1	14	2	3	15	21	9	0	328
Totals	464	449	545	541	566	342	531	347	414	307	228	316	416	342	437	447	421	252	389	397	557	332	291	307	289	∑9927

F O U R - The first cytogenetic map of the tuatara, *Sphenodon punctatus*

Authors:

O'Meally, D., Miller, H., Patel, H.R., Marshall Graves, J.A., Ezaz, T.,

Current status of paper (circle as appropriate):

Planned/In Preparation/Submitted/Under Revision/ Accepted /Published

Date paper accepted for publication or anticipated date of publication:

Early/Mid 2010

Name of journal:

Cytogenetic and Genome Research

Extent to which research is your own:

I collected all samples, performed all laboratory work and undertook all analyses.

Your contribution to writing the paper:

I wrote the draft of the paper, prepared figures and incorporated suggestions made by co-authors. I liaised with the journal editor and replied to the referee's comments.

If paper not yet accepted, has the paper been rejected by any journals:

No

Comments:

This paper was an invited contribution to a Special Issue on reptile cytogenetics and genomics. Material used in the study was made available as a result of an international collaboration initiated by myself with the second author.

Reviewer's comments and response

Review Form — *Cytogenetic and Genome Research*

Ms.No. 18 Editor A

I recommend that the manuscript entitled: **The first cytogenetic map of the tuatara, *Sphenodon punctatus***

by: **O'Meally et al.**

(1) ☒ be accepted for publication
- ☐ in its present form.
- ☒ after the **minor revisions** suggested below have been taken into consideration.

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☐ should be reviewed again after the indicated problems have been carefully considered and acted on by the authors.

- ☐ I would
- ☐ I would not welcome an opportunity to review the revised manuscript.

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(6) ☐ not be accepted for publication for the reasons indicated below.

Comments:

This is a very well-written paper on a topic of great interest for this special issue. I only have very minor suggestions for a few editorial changes for further clarification of the text, but other than those, the paper is nearly ready for publication.

Minor changes:

Abstract: Reword "this lonely species" – it is uncertain what you mean.

Now reads "this isolated species"

Page 84: What is the reasoning behind the statement that "it is likely that tuatara chromosome 4 is equivalent to the snake Z chromosome"? Expand this sentence to clarify.

This section has been expanded to clarify, with the addition of "Synteny and even the order of genes found on macrochromosomes are highly conserved among sauropsids. Genes spanning the length of the snake Z map to a contiguous block of chicken 2p and chromosome 6 of the butterfly lizard (Srikulnath et al., 2009b)."

Page 85. How do you rule out the alternative that tuatara chromosome 7 represent the ancestral state and that this ancestral chromosome generated chicken chromosome 9 and human 3 by fission? Please explain in the text.

This has been explained further in the text with the addition of "Fission of ancestral chromosomes could also explain this composition, though it would require a greater number of rearrangements."

Page An abnormal karyotype and tuatara microchromosomes87. How do you reconcile the observation of the absence of chromosome 17 in this individual which suggests that this microchromosome may contain no genes that are essential to life and the previous and later statement that microchromosomes are gene-rich chromosomes? Please discuss.

The text dealing with this topic under the subtitle *An abnormal karyotype and tuatara microchromosomes* has been reworded to clarify.

The first cytogenetic map of the tuatara, *Sphenodon punctatus*

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Abstract

Tuatara, *Sphenodon punctatus*, is the last survivor of the distinctive reptilian order Rhynchocephalia and is a species of extraordinary zoological interest, yet only recently have genomic analyses been undertaken. The karyotype consists of 28 macrochromosomes and 8 microchromosomes. A BAC library constructed for this species has allowed the first characterization of the tuatara genome. Sequence analysis of 11 fully sequenced BAC clones (~0.03% coverage) increased the estimate of genome wide GC composition to 47.8%, the highest reported for any vertebrate. Our physical mapping data demonstrate discrete accumulation of repetitive elements in large blocks on some chromosomes, particularly the microchromosomes. We suggest that the large size of the genome (5.0pg/haploid) is due to the accumulation of repetitive sequences. The microchromosomes of tuatara are rich in repetitive sequences, and the observation of one animal that lacked a microchromosome pair suggests that at least this microchromosome is unnecessary for survival. We used BACs bearing orthologues of known genes to construct a low coverage cytogenetic map containing twenty-one markers. We identified a region on chromosome 4 of tuatara that shares homology with 7Mb of chicken chromosome 2, and therefore the orthologous region of the snake Z chromosome. We identified a region on tuatara chromosome 3 that is orthologous to the chicken Z, and a region on chromosome 9 orthologous to the mammalian X. Since the tuatara determines sex by temperature and has no sex chromosomes, this implies that different tuatara autosome regions are homologous with the sex chromosomes of mammals, birds and snakes. We have identified anchor BAC clones that can be used reliably to mark chromosomes 3–7, 10 and 13, some of which are difficult to distinguish based on morphology alone. FISH mapping of 18S rDNA confirms the presence of a single NOR located on the long arm of chromosome 7, as previously identified by silver staining. Further work to construct a dense physical map will lead to a better understanding of the dynamics of genome evolution and organization in this isolated species.

Introduction

Tuatara have been described as the most distinctive surviving reptile in the world (Daugherty et al., 1990) and a species of extraordinary zoological interest (Groombridge, 1982). Molecular cytogenetic characterization and a gene map for the species would therefore be of great value for bringing this species into the genomic era. Tuatara are the only living representatives of the reptilian order Rhynchocephalia (also known as Sphenodontia), which diverged from other reptilian orders approximately 270 million years ago (Hugall et al., 2007). Rynchocephalids are regarded as the sister group of the squamates, based on morphological and genetic analyses (Rest et al., 2003; Hugall et al., 2007). They were globally widespread until the late Cretaceous (65-80 million years ago) but now survive only on some off-shore islands of New Zealand. Until recently, two allopatric species of tuatara were recognized: *Sphenodon punctatus* and *Sphenodon guntheri*, (Daugherty et al., 1990). However, recent genetic analyses by Hay et al. (2009) indicate that tuatara should be regarded as a single species (*Sphenodon punctatus*), with three distinct genetic/geographic groups.

In addition to their unique phylogenetic placement, several aspects of tuatara biology have the potential to inform studies of genome evolution and development. Tuatara are extremely long lived, probably surviving over 100 years (Dawbin, 1982; Castanet et al., 1988) and have a long generation time (sexual maturity at 14 years, mean generation interval 50 years; Cree et al., 1992; Allendorf and Luikart, 2006). Females reproduce only every four years on average (Cree et al., 1992), and a long period of egg incubation occurs prior to hatching (11-16 months in the wild). Tuatara have temperature dependent sex determination (TSD), and are one of the few species to exhibit a female-to-male pattern of TSD, where males are produced at high temperatures (Mitchell et al., 2006). No sex specific differences in chromosomes or DNA have been found (Norris et al., 2004; Mitchell et al., 2006). Tuatara also have unusual thermal biology, remaining active at extremely low temperatures (~5°C), and exhibiting the lowest optimal body temperature range of any reptile (16-21°C; Werner and Whitaker, 1978; Thompson and Daugherty, 1998; Besson, 2009). In accordance with this, they have a low metabolic rate (Thompson and Daugherty, 1998) and one of the largest reptilian genomes, with a C-value of 5.0 (Olmo, 2003, 1981; Vinogradov and Anatskaya, 2006).

Like most sauropsids, the tuatara karyotype is composed of macrochromosomes and microchromosomes. A complete karyotype of tuatara (2n=36, 28M & 8m) was first determined by Wylie et al. (1968), although earlier attempts had been made (Keenan, 1932; Hogben, 1921). Norris et al. (2004) provided the first comprehensive analysis of *Sphenodon* karyology,

including morphological descriptions of each chromosome, C-banding and silver staining of the nucleolus organizer (AgNOR). They examined 89 animals from across the range and reported no karyological differences between populations or between sexes. One animal from Ruamahua-iti Island possessed a peculiar chromosome 3, with additional material on the long arm of one member of the pair in all cells examined. To date, no other chromosomal aberration has been reported in this species.

On the basis of gross morphology of the macrochromosomes, Norris et al. (2004) suggested that the tuatara shares more similarity in karyology with turtles than with squamates. However, chromosome painting and gene mapping studies are much better able to reveal chromosomal homologies, and recent studies have shed light on the origin and evolution of sex chromosomes in particular (Graves, 2008). Chromosome painting has shown the chicken Z to be entirely homologous with chromosome 5 in *Chelodina longicollis*, an Australian turtle (Graves and Shetty, 2001). Chicken Z genes show homology to chromosome 6q in the Chinese soft-shelled turtle, *Pelodiscus sinensis*, and to chromosome 3 in the crocodile *Crocodylus siamensis*, (Kawai et al., 2007). Among squamates, chicken Z is equivalent to chromosome 2p of snakes (Matsubara et al., 2006) and 2p of two agamid lizards (Srikulnath et al., 2009a; Ezaz et al., 2009). Perhaps most remarkably, synteny, and even order, of six Z-borne genes in chicken and the gecko *Gekko hokouensis* Z chromosome is conserved (Kawai et al., 2009). The Z chromosomes of other reptiles are not as well characterized; however, the snake Z shares homology with chicken 2p and agamid 6p, and the turtle Z with chicken chromosome 15 (Matsubara et al., 2006; Ezaz et al., 2009; Srikulnath et al., 2009a; Kawagoshi et al., 2009). Given its unique phylogenetic position between archosaurs and squamates, tuatara is well placed to elucidate the mode of karyotype evolution among sauropsids.

The tuatara genome has received relatively little attention to date. Genetic studies have focused largely on the isolation of mitochondrial markers for phylogenetic and phylogeographical studies (Rest et al., 2003; Hay et al., 2003) and characterization of neutral (microsatellite) and adaptive (MHC) genetic markers for population analyses (Aitken et al., 2001; Miller et al., 2005, 2006; MacAvoy et al., 2007). Although more than 1,500 papers have been published on topics ranging from physiology to phylogeny of the tuatara, only four deal specifically with karyology and three with genomic organization. However, interest in tuatara genomics is increasing and the availability of a tuatara BAC library has recently enabled the first investigations of its nuclear genome (Wang et al., 2006; Shedlock, 2006; Organ et al., 2008). Here we present the first physical map of the chromosomes of tuatara and report our observations on genome evolution in this interesting and enigmatic reptile.

Materials and Methods

Animals, blood culture and chromosome preparation

Blood samples were collected from animals held captive at Taronga Zoo, Sydney (RFID implant numbers 6306A5 and F75DAE). Both specimens were female and originated from Stephens Is, New Zealand but were hatched in captivity in 1986 (M. Thompson, pers. comm.). Chromosomes were prepared from short-term culture of peripheral blood leukocytes. Up to 1mL of blood was collected by caudal venipuncture into heparinized tubes. The tubes were centrifuged at 260 x *g* for 5 minutes and the buffy coat collected. Cultures were established in 2mL of Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (JRH Biosciences), 1mg/mL L-glutamine (Sigma), 100 U/mL penicillin (Multicell), 100mg/mL streptomycin (Multicell) and 3% phytohaemagglutinin M (PHA M; Sigma). Cultures were incubated at 26°C for 6 – 7 days (Norris et al., 2004; Wylie et al., 1968) in 5% CO_2. Chromosomes were harvested 2 hours after adding colcemid (75ng/mL) by treating with hypotonic solution (KCl, 0.075mM) and fixation in methanol:acetic acid (3:1) according to standard protocols. The cell suspension was dropped on to slides and air dried. Slides were kept frozen at -80°C.

Probe selection, preparation and fluorescence in situ hybridization (FISH)

We mapped clones from a tuatara BAC library (Wang et al., 2006) made available commercially (SymBio Corp, Menlo Park, California). We chose clones that had been fully sequenced by the NIH Intramural Sequencing Center (www.nisc.nih.gov) as part of the NISC Comparative Sequencing Initiative. We also scanned the NCBI trace archives of paired BAC ends (Wang et al., 2006) for orthologues of genes found on chicken chromosomes Z and 2. The gene content of BAC clones was determined using BLAST after masking the query sequence using RepeatMasker (Smit et al., 2009). CG content was estimated as by Shedlock et al. (2007). We mapped the clones identified by Wang et al. (2006) as those containing *DMRT1*. To map 18S rDNA, we used a BAC clone containing this locus from the tammar wallaby (Haines, 2005). Some ambiguous assignments were clarified by comparing them with BACs of known location (H. Miller, unpub. data). Details can be found in Table 1.

Clones were grown in 15mL overnight cultures and BAC DNA extracted using the Promega Wizard Plus SV Miniprep DNA Purification System according to the manufacturer's protocol (with volumes scaled up). DNA concentration was measured on a NanoDrop (Thermo Scientific) and labeled by nick translation incorporating either Orange or Green-dUTP (Abbott Molecular). Labeled BAC DNA (200-500ng) and boiled genomic DNA (1µg) were co-precipitated and

resuspended in hybridization buffer (50% v/v deionized formamide, 10% w/v dextran sulphate, 2X SSC, 1X Denhardt's solution and 40mM sodium phosphate). Probes were added to slides under cover slips and sealed with rubber cement. DNA was denatured by heating the slide to 68.5°C for five mins on a heat block and hybridized overnight at 37°C in a humidified chamber. Cover slips were removed by soaking for 5 mins in 2X SSC. The slides were washed once in 0.4X SSC/0.3% IGEPAL CA-630 (Sigma) at 60°C for 2 mins, once in 2X SSC/0.1% IGEPAL at room temperature for 1 min, then dehydrated through an ethanol series (1 min in each of a 70%, 90% and 100% solution), air dried, stained with DAPI (50µg/mL DAPI solution in 2X SSC) for 45 sec at room temperature and mounted with Vectashield (Vector Laboratories). Vernier co-ordinates of each metaphase were recorded and images of 3-10 cells were captured using a Zeiss Axioplan2 epifluorescence microscope and images were analyzed using IPLab imaging software (Scanalytics Inc). Ambiguous chromosomal assignments were resolved by simultaneously hybridizing differentially labeled probes to a slide.

Telomere PNA probe

Telomeres were visualized following the protocol of Lansdorp et al. (1996) with some modifications. Briefly, 10µL of hybridization mixture containing 70% formamide, 0.3µg/mL Cy3-$(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Biosynthesis, Inc, Texas) and 1X Denhardt's solution in 10mM Tris pH 7.2 were added to the slide under a cover slip and sealed with rubber cement. The DNA was denatured by heating for 3 min at 80°C. After hybridization for 2 h at 37°C in a humidified chamber, the slides were washed at room temperature with 70% formamide/1% BSA/10mM Tris pH 7.2 (2 x 15 min) and then with 0.1M Tris/0.15M NaCl, pH 7.5 containing 0.08% Tween-20 (3 x 5 min). The slides were then dehydrated through an ethanol series, air dried, stained with DAPI and image capture and analysis was performed as described above.

Chromomycin A_3 (CMA_3) Staining

For CMA_3 staining, we used a modified CMA_3/methyl green method (Babu and Verma, 1995). 300µL of a solution containing CMA_3 (0.5mg/mL) and 5mM $MgCl_2$ in 0.5X McIlvaine's buffer (pH 7.0) was added to slides under a cover slip and incubated at room temperature for 1h. After brief rinsing in water, the slides were placed in a Coplin jar containing a freshly prepared solution of methyl green (100µM) in 0.1M Tris (pH 7.0) for 10 minutes. The slides were rinsed again in water, mounted as above and stored at 4°C for 1 week before image capture and analysis.

Silver staining (Ag-NOR)

Ag-NOR staining was carried out using the method of Howell and Black (1980). Briefly, 2 drops of a 2% gelatin/1% formic acid solution and 4 drops of a 50% $AgNO_3$ solution were added to a slide under a cover slip. The slide was incubated on a heat block at 70°C until the appearance of a golden brown color, then washed in distilled water and mounted as above. Images were captured under bright field microscopy.

Results

We chose 27 clones that contained orthologues of known genes, based on earlier full BAC sequencing, BAC end sequencing or library screening (Table 1, Fig 1). At least 21 genes were represented. We used fluorescence *in situ* hybridization (FISH) to assign these BACs unambiguously to eleven tuatara chromosomes (Fig 1). The tuatara karyotype contains sixteen pairs of macrochromosomes and four of microchromosomes. Some tuatara chromosomes are difficult to distinguish by size and morphology, particularly chromosome pairs 9 and 10, as well as 13 and 14 (Norris et al., 2004). We assigned BAC clones 475O6 to chromosome 9 and 531J19 to chromosome 13. The BACs we assigned to these chromosomes are useful as anchor markers because they produce single, clear signals that unambiguously identify the chromosomes. Similarly diagnostic clones for chromosomes 3–7 are identified in Table 1. Chromosomes 1–2, 8, 10–12 and 14 remain without anchor BACs, but can be distinguished by size and morphology. The four microchromosomes (15–18) can be distinguished by size and the hybridization pattern of two clones (515D6 and 437J19; Fig 2C).

Some fully sequenced BAC clones contain an unusually large number of unique repetitive elements amounting to ~90/Mb (Shedlock, 2006). We found that several BACs containing such repetitive elements hybridized to multiple chromosomes (Table1, Fig 2B, C & Fig 3D). For instance, the BAC clone 448I11 hybridizes to chromosomes 4, 6 and 13, in large domains which may represent up to 3% of the total chromosome length (TCL) (Fig 3D). Similarly, clone 437A11 hybridizes to 13q and to all four microchromosomes (~1%TCL). All clones identified by Wang et al. (2006) as containing *DMRT1* (Table 1) hybridize to both tuatara chromosomes 16 and 18 in a diffuse pattern, suggestive of a high repeat content in these chromosomes (~1%TCL). The smallest microchromosome, 18, appears to consist predominately of repeats contained in clone 437A11 and those associated with *DMRT1*.

Using an 18S rDNA FISH probe, we were able to co-locate the silver stained NOR and 18S rDNA hybridization signal, confirming the presence of a single active NOR on the distal end of the long arm of chromosome 7. In addition, in all cells examined, whether by AgNOR staining or by

FISH, one homolog consistently stained more heavily or gave a greater hybridization signal (Fig 3B). The PNA telomere probe produced clear and unambiguous hybridization signals identifying telomeres on each chromatid of all chromosomes (Fig 3C). No interstitial hybridization signals were observed on any chromosome. No general trend in arm length and signal intensity was apparent, but the microchromosomes appear to have disproportionately long telomeres.

To investigate the distribution of GC-rich sequences, we used CMA_3 methyl green staining (Fig 3A). No discernable bands were visible on any chromosomes, but more intense staining was invariably observed on the NOR. The microchromosomes also stained more heavily, indicative of a higher GC content in those elements. Using draft assemblies of fully sequenced BAC clones (Table 1; ~0.03% genome coverage) we estimated the genome wide GC content to be 47.76% (SD=0.63).

Of the two animals examined in this study, one animal (#6306A5) possessed a karyotype consistently deficient in one pair of microchromosomes (Fig 2). To investigate this further we took a second blood sample six months later and made new chromosome preparations. The PNA telomere probe (Fig 2A) revealed no intrachromosomal telomere sequence that could denote a fusion. Based on chromosome size and two-color hybridization with clones 437A11 and 515D6, each microchromosome pair is distinguishable. We identified the missing element as chromosome 17 by comparing the abnormal karyotype (Fig 2B) with that of a normal animal (Fig 2C).

Discussion

BAC-anchored cytogenetic map and chromosome homology

Even with this light coverage map, we were able to identify regions of conserved synteny between tuatara and other reptiles, as well as some rearrangements between tuatara and other species. The distal long arm of tuatara chromosome 4 corresponds to a 7Mb gene dense region of chicken chromosome 2, and is bounded by genes that are found on the snake Z chromosome (Matsubara et al., 2006). The synteny of this region appears to be conserved in all vertebrates but is interrupted in eutherian mammals between *GATAD1* (human 7q21) and *ARHGAP21* (human 10p12). Synteny and even the order of genes found on macrochromosomes are highly conserved among sauropsids. Genes spanning the length of the snake Z map to a contiguous block of chicken 2p and chromosome 6 of the butterfly lizard (Srikulnath et al., 2009b). Although no genes that have been located on the snake Z are

present on our map, it is likely that tuatara chromosome 4 is equivalent to the snake Z chromosome, which, in all snakes examined to date, is the fourth or fifth largest pair.

Tuatara 5q shares homology with a 4.7Mb region of chicken chromosome 5. Synteny of this block is conserved in all sequenced tetrapods with the exception of eutherian mammals (*GPHN* is on human 14q23 and *RTF1* on human 15q14). Two genes, *GRM8* and *SND1,* which in humans lie within 1.6Mb on chromosome 7, are respectively located medially and distally on the long arm of chromosome 7 in tuatara. Of the two genes contained in clone 460J16 in tuatara, *SND1* is absent from the chicken assembly but *RSRC1* lies on chicken chromosome 9 and human 3, suggesting that tuatara chromosome 7 arose by fusion of ancestral chromosomes. Fission of ancestral chromosomes could also explain this composition, though it would require a greater number of rearrangements. *PHF6* and *HPRT1* (contained in the same BAC clone) map to chromosome 9 in tuatara, indicating homology with a 46Kb region of chicken chromosome 4 and a 127Kb region of the human X.

The genes *ACO1* and *DMRT1* are located on the Z chromosome in chicken, but in tuatara the BAC clones corresponding to these genes map to chromosomes 3, 16 and 18 (Table 1). In all vertebrates studied to date, *DMRT* genes are arranged *DMRT1-DMRT3-DMRT2*: a region spanning 94Kb in zebra finch and up to 271Kb in opossum. To obtain tuatara clones containing *DMRT1*, Wang et al. (2006) screened the BAC library using conserved intronic and intergenic regions from *DMRT1* and DM domain sequences of *DMRT2*. They assembled ten BACs into a 300Kb contig from which they were able to amplify the non-coding probe sequences, but failed to amplify the DM domain of any gene. This suggested to the authors that they had not identified *DMRT1* but had instead found a duplicated cassette of the non-coding regions. Hybridization of each of the ten contiguous BAC clones to two chromosomes in a diffuse pattern strongly supports this suggestion, and our mapping of *DMRT1* to chromosomes 16 and 18 should be considered provisional.

Repeat content and GC composition

Our physical mapping data demonstrate discrete accumulation of repetitive elements in large domains on some chromosomes, particularly the microchromosomes. Three of the clones we mapped (437A11, 448I11, 515D6) account for approximately 5% of the total chromosome (genome) length (Figs 2C, 3D). Tuatara has a large genome (5.0pg/haploid; Olmo, 1981) with more than twice the number of repeat types per megabase than *Anolis*; indeed, more than twenty times the zebra finch (Shedlock, 2006), yet the gene structure (exon and intron length) is much the same as for birds and other reptiles (Organ et al., 2008). Genome survey

sequencing of 89 BAC ends (~121Kb; Wang et al., 2006) and analyses of 11 fully sequenced BACs (1.6Mb; Table 1; Shedlock, 2006) suggest that retroelements make up about 5–6% of the tuatara genome. By comparison, transposable elements make up about 9% of the minimal chicken genome and 40–50% in humans (ICGS Consortium, 2004; Lander et al., 2001). A figure of 6% for tuatara must therefore be a gross underestimate of the true value, due to the low proportion (~0.03%) of the genome sampled and the poor representation of reptile repeats in databases used by RepeatMasker (Chapus and Edwards, 2009). The tuatara's large genome, then, is almost certainly due to accumulation of repetitive DNA.

We used CMA_3-methyl green staining to visualize GC isochore structure on tuatara chromosomes. Previous attempts using G-banding have not been successful (Norris et al., 2004; and in our laboratory). Notably, all microchromosomes and the NOR on the long arm of chromosome 7 were densely stained by CMA_3. This suggests that the microchromosomes have a higher GC content than the rest of the genome, as is the case in chicken and soft-shelled turtle (Auer et al., 1987; ICGS Consortium, 2004; Kuraku et al., 2006). A high GC content on the microchromosomes implies an increased recombination rate (Marsolier-Kergoat and Yeramian, 2009; Fang et al., 2008) and also correlates with increased gene density (Costantini et al., 2006, 2007). The lack of isochore structures on the macrochromosomes reveals a fairly homogeneous GC distribution on these chromosomes in tuatara, as is the case for many other reptiles, fish and amphibians (Bernardi, 2000; Hughes et al., 2002). The nucleolus organizer region, containing 18S rDNA, is GC rich in all animals (Varriale et al., 2008) so it was not surprising to see dense staining of CMA_3 in this region. Despite a homogenous GC distribution, the tuatara genome is composed of 47.76% GC (SD=0.63), the highest known for any vertebrate (Table 2, Costantini et al., 2009; Olmo, 2008). This estimate of GC content is based on our analysis of 11 fully sequenced BAC clones representing only about 0.03% of the genome, so it may be an unrepresentative sample and may change as more in-depth sequence data become available for tuatara.

Telomeres

Telomere length has been found to be negatively correlated with age in snakes (Bronikowski, 2008), turtles (Hatase et al., 2008), alligators (Scott et al., 2006), birds (Haussmann and Vleck, 2002; Haussmann et al., 2003), humans (Tsuji et al., 2002) and in many other taxa, so as a species that is thought to live for over 100 years (Dawbin, 1982; Castanet et al., 1988) tuatara present a good model for examining telomere length and senescence. Indeed, by comparison with other vertebrates, tuatara appear to have relatively long telomeres (H. Bender pers. comm.). Quantitative methods such as QFISH (Zijlmans et al., 1997;

Slijepcevic, 2001) or qPCR (Cawthon, 2002; O'Donovan et al., 2009) are needed to confirm this observation. Long telomeres have been found in many long-lived vertebrates, including turtles (Hatase et al., 2008), birds and some mammals (Haussmann et al., 2003). Some studies have suggested a positive correlation between telomere length and total chromosome or arm length (Wise et al., 2009; Sridevi et al., 2002; Zijlmans et al., 1997); however, our results do not support this observation. In tuatara, the size of microchromosomal telomeres is equivalent to or longer than those of the macrochromosomes (Figs 2A, 3C). Expansion of the telomeric sequences may be due to the repetitive content of the microchromosomes.

Interstitial telomeric repeat sequences associated with constitutive heterochromatin (as revealed by banding techniques) may be indicative of ancestral chromosomal rearrangements in some vertebrates (Meyne et al., 1990; Ruiz-Herrera et al., 2008). Vague C-banding, no demonstrable G-bands (Norris et al., 2004) and the absence of interstitial telomeric signals in all chromosomes suggest that the tuatara has a relatively stable and evolutionarily derived karyotype (Meyne et al., 1990; Nanda et al., 2002; Ruiz-Herrera et al., 2008). Having no obvious interstitial telomeric sequences also makes this species amenable to a rapid and reliable qPCR test for telomere length (Nakagawa et al., 2004). Determining the age of tuatara once they reach adulthood is currently problematic, and a lack of long-term (i.e. >50 year) longitudinal studies of marked animals means that many aspects of tuatara life history (such as maximum longevity, the age structure of populations and at what age they cease to be reproductively active) are unknown. Conservation management of tuatara would therefore benefit greatly from such an assay if a relationship between age and telomere length could be demonstrated.

An abnormal karyotype and tuatara microchromosomes

Our study identified a female with an abnormal karyotype that was consistently lacking in one pair of microchromosomes. The hybridization pattern of two BAC clones (437A11 and 515D6) differs between the odd animal and an animal possessing a normal chromosome complement only at chromosome 17, suggesting loss rather than fusion with another chromosome. If the disappearance of the chromosome had resulted from fusion with another pair, interstitial telomeric signals might have been observed, but they were not. This loss of a microchromosome is a particularly striking finding because the animal still survives and is apparently healthy at 23 years of age (although the affect on its reproductive capacity remains unknown). Chromosome 17 represents about 1-1.5% of the genome (Norris et al., 2004) but presumably it carries largely repetitive sequences and contains no genes that are essential for survival. The higher GC composition of microchromosomes may be due to the frequency of

CR1-like LINE repeats and a greater recombination rate, rather than higher gene density in these chromosomes.

Although fewer, the four microchromosomes of tuatara are similar in many respects to those of birds and other reptiles. Our mapping data indicate a high repeat content on all microchromosomes, particularly chromosomes 17 and 18, explaining the viability of one animal which lacks chromosome 17. The microchromosomes of birds and turtles are also repeat rich (Stefos and Arrighi, 1974; Matzke et al., 1990; Fillon et al., 1998; Yamada et al., 2005) despite their abundance of genes (McQueen et al., 1998; Burt, 2002; Kuraku et al., 2006). The size of tuatara microchromosomes relative to the rest of the genome (smallest 0.9% - largest 2%; Norris et al., 2004) is comparable to the range found in chicken (smallest 0.4% - largest 2%; Bloom et al., 1993). In absolute terms, however, the microchromosomes of tuatara are very much larger, with a size range of 43Mb–113Mb (1pg=978Mb; Dolezel et al., 2003), compared to just 7Mb–23Mb in chicken (Bloom et al., 1993). The gene content of these large tuatara microchromosomes remains unknown, and we were unable to examine replication timing explicitly. Squamates and archosaurs (with the exception of crocodilians) usually possess a large complement of microchromosomes. The reduction of microchromosomes in the tuatara karyotype probably proceeded by fusion events, as proposed for crocodilians and many birds (Burt, 2002; de Oliveira et al., 2005; Nie et al., 2009).

Conclusion

Tuatara has frequently been referred to as a 'living fossil' because it bears some skeletal features mistakenly interpreted as primitive (they are in fact derived) (Whiteside 1986; Mo et al., 2009). It appears that many features of its genome are evolutionarily derived as well. These include the large genome size (Thomson and Muraszko, 1978; Burt, 2002), a high GC content (Wang et al., 2006), absence of interstitial telomeres (Meyne et al., 1990) and a small number of microchromosomes (Norris et al., 2004). Our study presents a preliminary overview of genome evolution and organization in tuatara. A gene dense cytogenetic map covering all the chromosomes will elucidate the extent to which lineage specific chromosomal rearrangements have occurred, and which are retained. Such a map is also essential should a tuatara genome project be undertaken (Lewin et al., 2009). Even low coverage sequencing can be of great utility (Green, 2007) and the tuatara's large genome may harbor countless novel genes and regulatory elements (Peterson et al., 2009).

Our low-coverage cytogenetic map identified a region on chromosome 4 that shares homology with chicken chromosome 2, and therefore the orthologous region of the snake Z

chromosome. We identified a region on chromosome 3 that is orthologous to the chicken Z, and a region on chromosome 9 homologous to the mammalian X. A cytogenetic map of higher resolution would be of great benefit in understanding the evolution of amniote sex chromosomes from autosomal pairs. Similarly, knowledge of the genic content of the microchromosomes will lead to a greater understanding of their evolution; our observation of one animal that lacked a pair suggests that not all microchromosomes are necessary for survival. Sequence analysis of a small genome sample increased the estimate of genome wide GC composition to 47.8%, the highest reported among vertebrates to date. This is an interesting observation as high GC content has often been considered an adaptive response to high body temperature (Bernardi, 2000; Olmo, 2003), but tuatara has the lowest active body temperature of any amniote. The tuatara's large genome is doubtless due to accumulation of repetitive sequences. Such sequences are key agents of regulatory innovation, chromosome rearrangements and evolutionary change (Sharp et al., 2006; Jurka et al., 2007). Perhaps this species' generous endowment of repetitive DNA has played a role in its continued survival over the last 60MY, despite dramatic changes in geology, climate and population size (Cooper and Cooper, 1995; Landis et al., 2008; Jones et al., 2009).

Acknowledgements

We are grateful to Taronga Zoo for access to animals in their collection and to Hannah Bender for her assistance with the telomeric probe. Samples were collected under Taronga Zoo AEC protocol 3b/04/07.

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Tables and Figures

Table 1. Details of BAC libraries and clones mapped. The genic content of fully or end sequenced clones was determined by BLAST after having masked the sequence for vertebrate retroelements, DNA transposons, simple repeats and low complexity motifs using RepeatMasker. Clones that uniquely identify chromosomes (anchor BACs) are indicated by an asterisk. The 10 clones thought to contain *DMRT1* were mapped both in pools (simultaneously) and individually with the same hybridization pattern obtained.

Library ID	Reference	Clone ID	Sequence accession	Representative loci	Chromosomal location
AGI, *Macropus eugenii* (tammar wallaby)					
	Haines, 2005				
		329J14*		18S rRNA	7q
VMRC12, *Sphenodon punctatus* (tuatara)					
	Wang et al., 2006				
		69A1*	gnl|ti|908600346	ACO1	3p
		515D6;162F5; 289C19; 557I22; 497H14; 82P24; 150B6; 269F20; 81B4; 58N13		DMRT1	16;18
	NISC Comparative Sequencing Initiative				
		40N7	AC154075	T	3q
		224G8	AC155214	ARHGAP21	4q
		239M1	AC153105	PPP1R9A; PON2	4q
		311J16	AC153757	GATAD1;ANKIB1	4q
		16O14*	AC154160	GPHN	5q
		462H11	AC154989	RTF1; NDUFAF1	5q
		178H23	AC155213	NOTCH1	6q
		436B16*	AC155215	INPP5E; NOTCH1	6q
		356P22	AC154074	GRM8	7q
		460J16	AC153758	RSRC1; SND1	7q
		475O6*	AC161716	PHF6; HPRT1	9p
	H. Miller (unpublished data)				
		44B3*		MHC-related	4q
		531J19*		MHC-related	13q
		437A11		MHC-related	13q;15–18
		448I11		MHC-related	4q;6p;13q

Table2. Genome-wide GC composition of representative amniotes. The tuatara has the highest GC content of any vertebrate reported to date. Data are from this study, Shedlock et al. (2007) and Warren et al. (2008).

Species	%GC
Human	39.9
Platypus	45.5
Chicken	40.2
Alligator	42.5
Turtle	43.6
Anolis	41.5
Tuatara	47.8

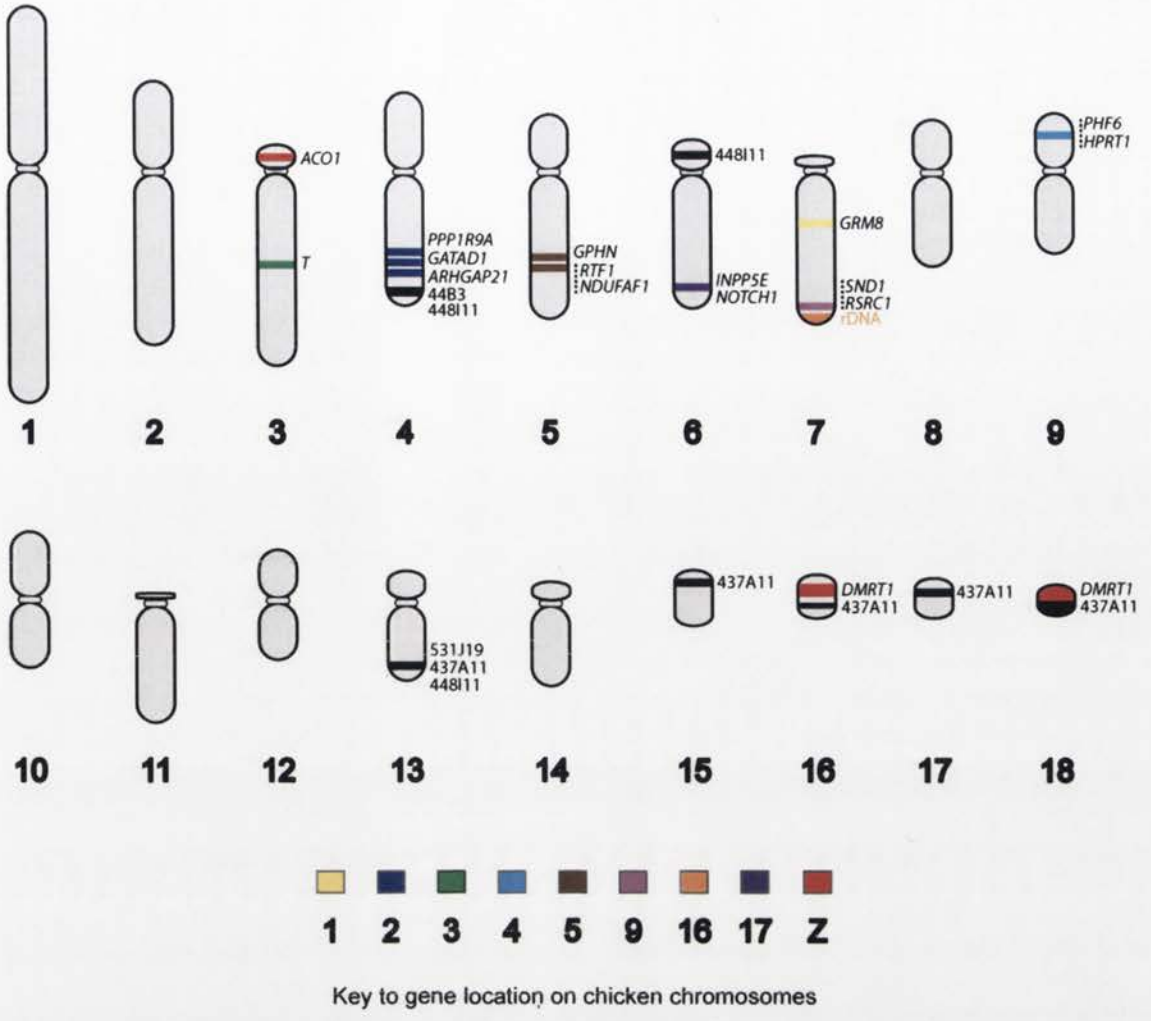

Figure 1. Tuatara–chicken comparative gene map. Fully sequenced BAC clones were mapped by FISH to metaphase chromosomes and their gene content determined by BLAST, or by library screens. Colors indicate homology to chicken chromosomes. Clone numbers are indicated in black where no genic data were available. Gene symbols are those recommended by the HUGO Gene Nomenclature Committee. Gene symbols joined by a dashed line indicate loci found in the same BAC clone but whose order on the chromosome is unknown.

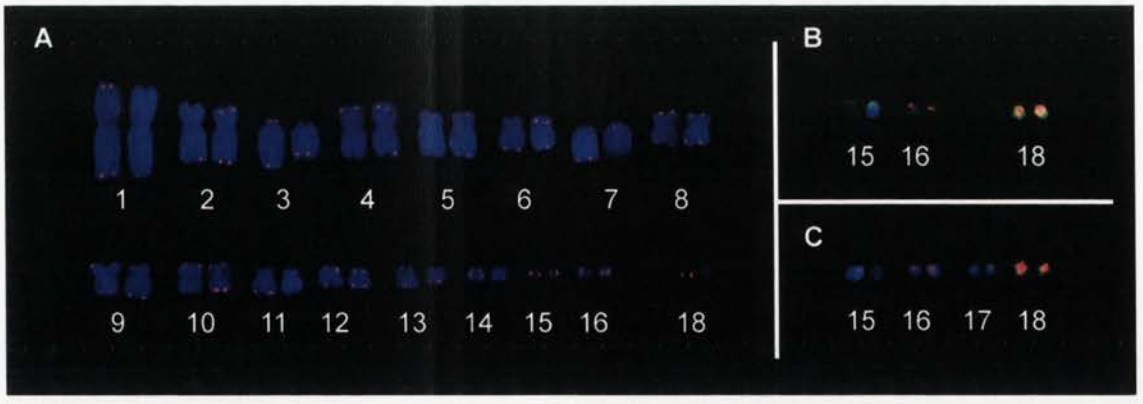

Figure 2. (**A**) Karyotype of animal #6306A5, lacking the third largest pair of microchromosomes (chromosome 17). The telomeric probe reveals only four signals for each chromosome, indicating that the loss of this pair is not the result of a fusion event. (**B**) Using differentially labeled BAC clones (515D6 in red and 437A11 in green) that hybridize to all microchromosomes, we were able to determine that the 17th pair was missing by comparison with the karyotype of an animal with a full chromosomal complement (**C**).

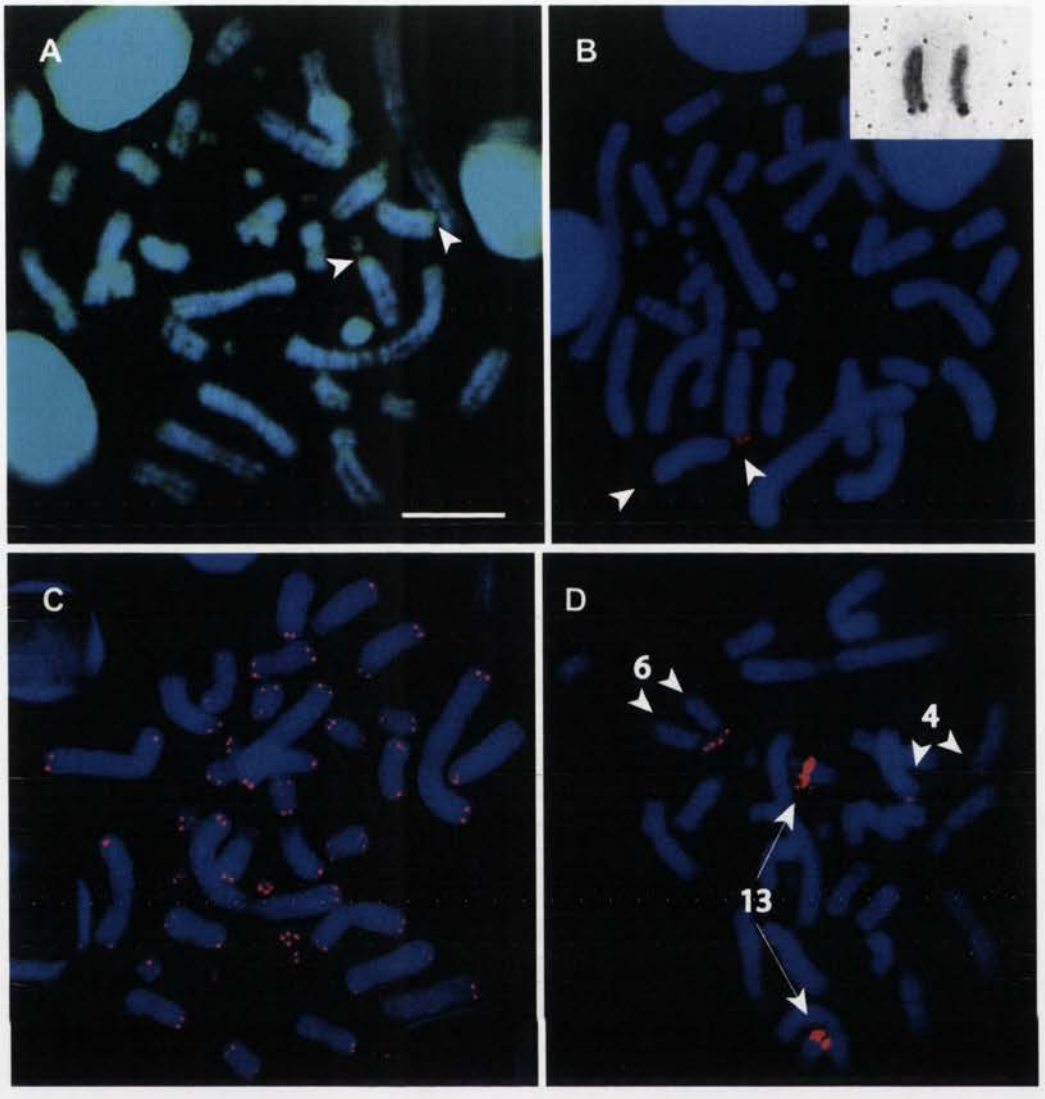

Figure 3. (**A**) Chromomycin A_3 and methyl green staining of tuatara metaphase chromosomes. Arrows indicate the position of the NOR on chromosome 7, which is more heavily stained. Staining of the microchromosomes is also marginally more intense. (**B**) FISH mapping of 18S rDNA to chromosome 7 (arrowheads) is concordant with AgNOR staining (inset). (**C**) Telomeres are clearly visible on each chromosome and no interstitial signals were found, suggesting no recent rearrangements. (**D**) Some clones (eg 448I11) hybridize in a pattern consistent with discrete accumulation of repetitive sequences in large domains on multiple chromosomes. The scale bar represents 10μM.

F I V E - A simple non-invasive protocol to establish primary cell lines from tail and toe explants for cytogenetic studies in Australian dragon lizards (Squamata: Agamidae)

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Current status of paper (circle as appropriate):

Planned/In Preparation/Submitted/Under Revision/Accepted/ Published

Date paper accepted for publication or anticipated date of publication:

January 2009

Name of journal:

Cytotechniques **58**: 135-139

Extent to which research is your own:

I collected samples for the study, initiated cell lines and made chromosome preparations jointly with my supervisor (T. Ezaz)

Your contribution to writing the paper:

The draft of this paper was jointly prepared by my supervisor and me. I prepared figures and edited several drafts.

If paper not yet accepted, has the paper been rejected by any journals:

N/A

Comments:

This paper reports the methods developed in our laboratory by me and others for the preparation of cell lines from reptiles using non-invasive, non-lethal techniques.

Cytotechnology (2008) 58:135–139
DOI 10.1007/s10616-009-9182-3

BRIEF REPORT

A simple non-invasive protocol to establish primary cell lines from tail and toe explants for cytogenetic studies in Australian dragon lizards (Squamata: Agamidae)

Tariq Ezaz · Denis O'Meally · Alexander E. Quinn · Stephen D. Sarre · Arthur Georges · Jennifer A. Marshall Graves

Received: 17 October 2008 / Accepted: 20 January 2009 / Published online: 6 February 2009

Abstract Primary cell lines were established from cultures of tail and toe clips of five species of Australian dragon lizards: *Tympanocryptis pinguicolla*, *Tympanocryptis* sp., *Ctenophorus fordi*, *Amphibolurus norrisi* and *Pogona vitticeps*. The start of exponential cell growth ranged from 1 to 5 weeks. Cultures from all specimens had fibroblastic morphology. Cell lines were propagated continuously up to ten passages, cryopreserved and recovered successfully. We found no reduction in cell viability after short term (<6 months) storage at −80 °C. Mitotic metaphase chromosomes were harvested from these cell lines and used in differential staining, banding and fluorescent in situ hybridisation. Cell lines maintained normal diploidy in all species. This study reports a simple non-invasive method for establishing primary cell lines from Australian dragon lizards without sacrifice. The method is likely to be applicable to a range of species. Such cell lines provide a virtually unlimited source of material for cytogenetic, evolutionary and genomic studies.

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Keywords Reptiles · Lizards · Cell lines · Tail · Toe · Non-invasive · Chromosomes

Introduction

Australian dragon lizards (Agamidae) are an ideal group in which to study sex chromosome evolution because of their recent radiation from an Asian ancestor (~25 million years ago; Hugall et al. 2008) and because there appears to have been multiple transitions between genotypic and temperature-dependent sex determination (Ezaz et al. 2009; Harlow 2004; Sarre et al. 2004). There are about 70 species in 13 genera (Cogger 2000). Although karyotypes have been described for many species, about 60% remain unexplored (Olmo 2005). In our laboratory, we routinely establish short term blood culture and primary fibroblast cell lines from internal connective tissue for larger species of agamid. From these we harvest chromosomes and interphase nuclei, and extract nucleic acids (DNA, RNA) for molecular and cytogenetic studies. However, some species cannot be sacrificed as they are listed as endangered or vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Australia). Many species are too small to obtain sufficient volumes for blood culture and would otherwise require invasive or post mortem sampling.

Explant culture is a routine procedure for establishing fibroblast-like cell lines from various organs,

and it has been optimised in various animals and used in a wide variety of biological experiments (Freshney 2006; Masters and Stacey 2007). Cell lines established from explant culture provide an almost unlimited source of chromosomes, interphase nuclei, DNA, RNA and other biomolecules, which can then be used in a wide variety of genomic and evolutionary studies. However, use of internal organs as explant for the establishment of primary fibroblast cell lines requires euthanizing animals, an option that is often not available in endangered and threatened species. An alternative approach to obtaining metaphase chromosomes is to set up short term blood culture but that too can be difficult in small species where the collection of sufficient blood necessary to establish successful cultures is problematic or when bleeding may lead to infection and stress to animals. A non-invasive approach would clearly be of value in such cases.

Culture of tail and epidermal explants has been reported in one lizard (*Anolis carolinensis* Simpson and Cox 1967); and a turtle (*Chelonia mydas* Mansell et al. 1989); while caudal fin explant has been used to establish primary cell lines in channel catfish (Zhang et al. 1998). However, the majority of reported studies used either embryonic tissues or various organs collected post mortem (see Mansell et al. 1989 and references therein).

In the current study, we developed a non-invasive technique to establish primary fibroblast cell lines from explant culture of tail and toe clips, and optimised culture conditions and cryopreservation of these cells lines. We characterized the cell lines by harvesting metaphase chromosomes and subsequent cytogenetic analysis.

Methods

Animals

Wild animals were collected from various locations across Australia. Mallee dragons (*Ctenophorus fordi*) were collected from Yathong Nature Reserve (32°S 145°E), Norris' dragons (*Amphibolurus norrisi*) were collected from Big Desert National Park (35°S 141°E), grassland earless dragons (*Tympanocryptis pinguicolla*) were collected from Canberra (35°S 149°E), and *Tympanocryptis* sp. (species not determined) were collected from Corella Creek Station (19 21°S × 136 02°E) and central bearded dragons (*Pogona vitticeps)* were collected from Murray-Sunset National Park (34°S 141°E).

Reagents and culture medium

Washing Buffer (WB): 1× PBS (calcium–magnesium free, pH 7.2–7.4 with phenol red). Collection Medium (CME): prepared inside a laminar hood by supplementing plain DMEM (Gibco) with 1 mg/mL kanamycin (Sigma), 40 μg/mL chloramphenicol (Sigma), 60 μg/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco). This mix was sterilized by passing through a 0.2 μm filter (Millipore GP). Subsequently, amphotericin B (Sigma) and tetracycline chloride (Sigma) were added to a final concentration of 20–100 μg/mL, respectively. We have found collection medium can be stored in 50 mL aliquots for up to 6 months at −20 °C. Culture Medium with Antibiotics (CMA): Amniomax C-100 (basal medium and supplement, Gibco), 100 U/mL penicillin G, sodium salt (Gibco), and 100 μg/mL streptomycin sulfate (Gibco). Trypsin Solution (TS): 0.1% w/v in 1× PBS, 0.1% w/v tetrasodium EDTA (Sigma). Freezing Medium (FM): 20% foetal bovine serum (Invitrogen) and 10% DMSO (Sigma) in plain DMEM. Hypotonic Solution (HS): 0.075 mM KCl. Fixative (FIX): 3 parts methanol, 1 part acetic acid.

Tissue collection

The surfaces of the tail tips and toes of adult dragon lizards were sterilized by wiping with gauze soaked in 70% ethanol. Approximately 5–10 mm of the tail tip was collected from each specimen using a sterile scalpel blade (*A. norrisi*, one male, one female; *C. fordi*, three females, *T. pinguicolla*, one male, *Tympanocryptis* sp. one male and *P. vitticeps*, one female). Three millimetres of the fourth toe of the hind foot were collected from a single male *A. norrisi* specimen. Tail and toe clips were immediately transferred to 5 mL of CME and incubated at room temperature (~25 °C) for 12–24 h.

Cultures

All explants for cultures were set up in an aseptic environment inside a laminar hood using sterilised

equipment. Individual tissue pieces were placed onto a Petri dish and washed twice with 1× WB. These were then transferred to a new Petri dish containing two drops plain DMEM and minced with scalpel blades. Culture flasks were prepared by scratching the inner surface with a scalpel blade (to assist in attachment of explants) and coated with 2 mL of plain DMEM, which aids later spreading of the medium without dislodging explants. Minced tissue pieces were transferred using a Pasteur pipette to a T25 culture flask and left to dry upright, with the lid open, for 30–60 min inside the hood after which 5 mL of CMA were added. Flasks were then transferred to a humidified incubator at 28 °C with 5% CO_2.

Splitting and freezing cultures

Cells were passaged once high density cell growth around primary explants was observed. Each flask was rinsed once with 3 mL 1× WB. Then, following the addition of 1.5 mL of TS, the flask was incubated at RT for 1–2 min or until cells were dislodged from the flask surface (with gentle tapping and observation under a light microscope) and mixed gently with a Pasteur pipette to remove cell clumps. To collect the cells, 5 mL (~3 volumes of TS) of CMA was added to the flask and transferred to a 10 mL tube. The cell suspension was centrifuged at 250*g* in a swivel rotor centrifuge for 5 min and the supernatant discarded, leaving 100–200 μL behind. The resultant cell pellet was then resuspended in 2 mL of CMA and mixed gently by tapping and then transferred to a new flask containing 3 (for T25) or 8 (T75) mL of CMA. Flasks were incubated as described above.

For freezing, a cell pellet was obtained as above, but resuspended in 1.5 mL FM, transferred to a chilled cryovial (Nunc) with appropriate labelling and stored at −80 °C for at least 2 days. For long term archival storage, vials were transferred to a liquid N_2 storage facility. We found cells were viable when stored at −80 °C for up to 6 months.

Chromosome preparations, differential staining, banding, FISH and microscopy

Metaphase chromosomes were harvested as described by Ezaz et al. (2005) with minor modifications. Specifically, cells were grown in culture until there was a high proportion of dividing cells (~50% with 60–70% overall confluence). The dividing cells are normally rounded and appear as doublets. Cell divisions were blocked at metaphase by adding 0.05 μg/mL of Colcemid (Roche) and incubated as described earlier for up to two hours, checking under a microscope every half an hour. When cells respond to colcemid (with high frequency of rounded cells), the medium was discarded, trypsinized, centrifuged and aspirated as described above. The pellet was resuspended gently and 1 mL HS added drop by drop. Another 2 mL of HS was then added slowly, mixed gently and incubated in a 37 °C water bath for 45–60 min. Several drops of freshly prepared chilled fixative were added and the cells centrifuged as before. The supernatant was removed except for 1 mL in which the pellet was resuspended. The cells were fixed by slowly adding 1 mL FIX. Another 1 mL of FIX was added and centrifuged as before. The cell pellet was washed three more times and finally FIX was added on the basis of the size of the pellet. The quality and mitotic index of the cell suspension was examined by dropping cells onto microscope slides and observing under a phase contrast microscope. Fixed cell suspensions were kept at −20 °C for long-term storage. Differential staining (DAPI, CA3, banding (C-banding), FISH with telomeric probe (TTAGGG)n and microscopy were performed as described by Ezaz et al. (2005).

Results and discussion

We have presented techniques to establish, propagate, maintain and archive primary fibroblastic cell lines from toe and tail clip explants of Australian dragon lizards, without the need to sacrifice animals.

Cells started to grow out from the explants within 4–5 days and all primary explants yielded dividing cells. The time required for initiation of exponential cell growth (~40–70% confluency) was variable, ranging from 1 to 5 weeks. In all species, explants produced primary cell lines with fibroblastic morphology (Fig. 1a). Cell lines were maintained and propagated through up to ten passages and cryopreserved successfully. After each passage, 70–80% confluency was reached within 1–3 days (Fig. 1a), after which cells were subcultured at at a 1:1 ratio. Cells were also subcultured at 1:3 and 1:4 ratios and

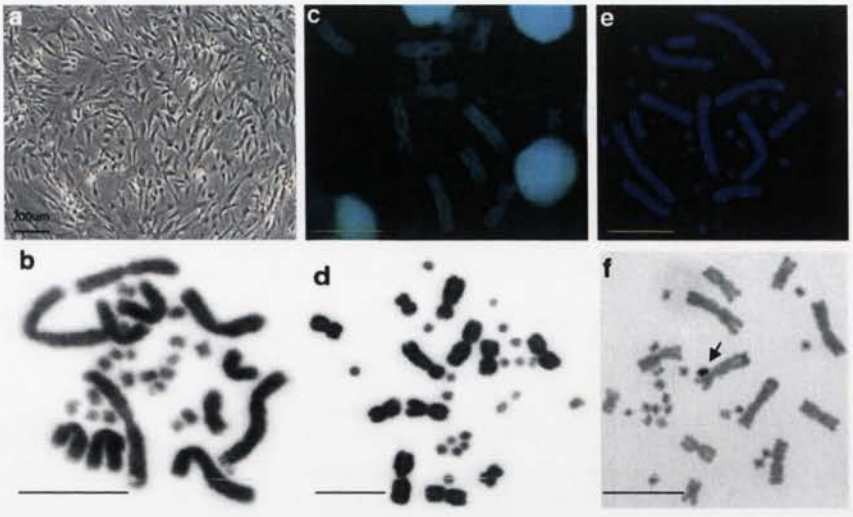

Fig. 1 Examples showing fibroblast cells and chromosomes harvested from tail explants of Australian dragon lizards. **a** fibroblast cell line obtained from tail clip of *T. pinguicolla*; **b** inverted DAPI stained chromosomes in *T. pinguicolla*; **c** merged images of CA3 and DAPI stained chromosomes in *A. norrisi*; **d** inverted DAPI stained images in *C. fordi*; **e** FISH of telomeric probe in *P. vitticeps*; **f** C-banded chromosomes of female *P. vitticeps*, *arrow indicates* C-banded W chromosomes. *Scale bar* represents 10 μm

50–70% confluency was reached within 1–3 weeks. No substantial mortality was observed when subcultured at the lower innoculi. The medium was changed once a week but no significant mortality was observed when the medium was changed only after 2 weeks. We found no reduction in cell viability after short term (<6 months) storage at −80 °C. We found no mortality in response to trypsin in any of our cell lines and no reduction of growth after recovery from cryopreservation.

To optimise culture conditions we varied the composition of the culture medium, incubation temperature and maceration versus enzymatic (collagenization) methods of primary cell isolation (data not shown). We compared explant cultures in 10% DMEM and 1:1 ratio of 10% DMEM and Amniomax to that of cultures with Amniomax only. We found optimum cell growth when cultured in Amniomax only. We incubated cultures at three temperatures: 26, 28 and 30 °C. We observed optimum cell growth in cultures incubated at 28 °C. We have also compared collagenization of explants with the maceration technique described herein. We found high cell mortality as well as frequent bacterial and fungal infections when collagenization was used. We observed no fungal or bacterial infection when our maceration technique was used.

Metaphase chromosome preparations were obtained from all cell lines reported here (Fig. 1b–f). The diploid chromosomes in Australian dragon lizards are highly conserved with the majority of species (including the five species in this study) having a chromosome complement of $2n = 32$ comprising 10 macro-chromosomes and 12 micro-chromosomes (Witten 1983). Our cell lines maintained normal chromosomal diploidy throughout the study. Chromosomes from all five species were used successfully in various cytogenetic analyses such as differential staining (DAPI, CA3, C-banding; Fig. 1b–d), C-banding (Fig. 1f) and fluorescent in situ hybridisation (Fig. 1e). We found no differences in banding, staining or FISH pattern when chromosomes were harvested from tail or toe clip fibroblasts when compared to those harvested from leukocytes or fibroblast cell lines originated form internal tissue culture. Of particular note are the chromosomes from *T. pinguicolla*, an endangered species and *A. norrisi* for which these preparations represent the first reported metaphase chromosomes and diploid chromosome numbers ($2n = 32$).

The protocol presented here is less labour intensive than traditional methods and could be easily adopted by any laboratory equipped for basic cell culture. Lizard toe and tail clips are routinely collected from wild animals in the course of ecological, evolutionary, taxonomic and conservation studies.

Establishing primary cell lines from external implants as we have demonstrated here, maximises the utility of samples that are often rare, expensive and difficult to collect, particularly in the case of endangered species. We have shown that primary cell

lines can be established from a very small (~3 mm) tissue explant without laborious trypsin or collagenase treatments. We are optimistic that this non-lethal technique can be used in other reptiles with appropriate modifications and, therefore, will provide research materials not only in cytogenetic studies but a wide variety of biological research.

Acknowledgments We thank Wendy Dimond and Tobias Uller for tissue samples of *T. pinguicolla* and *C. fordii*, respectively. Riccardo Natoli for cell photography. Animal collection, handling, and sampling were performed according to the guidelines of the Australian Capital Territory Animal Welfare Act 1992 (Section 40) and licences issued by the Queensland, South Australia, Victoria, Australian Capital Territory, and New South Wales state governments. All experiments were performed with the approval of the University of Canberra Animal Experimentation Ethics Committee (Proposal CEAE 04/04) and the Australian National University Animal Experimentation Ethics Committee (Proposals R.CG.02.00 and R.CG.08.03). This work is supported by Australian Research Council Discovery Grants (DP0449935 and DP0881196) awarded to Jenny Graves, S·S. and A.G., and S·S. and A.G, respectively.

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S I X - Sex chromosome evolution in lizards: independent origins and rapid transitions

Authors:

Ezaz, T., Sarre S., **O'Meally, D.**, Georges, A., Marshall Graves, J.A.

Current status of paper (circle as appropriate):

Planned/In Preparation/Submitted/Under Revision/ Accepted /Published

Date paper accepted for publication or anticipated date of publication:

Early/mid 2010

Name of journal:

Cytogenetic and Genome Research

Extent to which research is your own:

This is a review paper. I provided input to the major conclusions of this review .

Your contribution to writing the paper:

I contributed to and edited several drafts of the manuscript and prepared figures.

If paper not yet accepted, has the paper been rejected by any journals:

No

Comments:

This paper was an invited contribution to a Special Issue on reptile cytogenetics and genomics.

Reviewer's comments and response

Title: Sex chromosome evolution in lizards: independent origins and rapid transitions
Authors: Ezaz T., Sarre S.D., O'Meally D., Marshall Graves J.A.M. and Georges A.

This manuscript by Ezaz et al. reviews the sex chromosomal evolution and sex determining systems in lizards based on many references including the latest ones and their recent achievements, and discusses multiple transitions between TSD and GSD and between XY and ZW heterogamety, especially focusing on an association between the occurrence of female heterogamety and TSD within lizard families. The authors emphasize their conclusion that the lizard sex determination may be more the result of an interplay between sex chromosomes and temperature than previously thoughts. In this review paper, the distribution of GSD and TSD, male and female heterogamety and sex chromosomal differentiation stage in each family of Lacertilia are well summarized, and their statements on the sex chromosomal evolution and sex determining systems in lizards are suggestive. This paper is appropriate for the CGR special issue on reptilian cytogenetics and genomics. However, several points should be considered before publication.

Comments

1. Transition from female heterogamety to male haterogamety is readily explicable by the occurrence of a male dominant sex determining gene on autosomes, which overrides the female heterogametic sex determination system, followed by the disappearance of the female-specific chromosome. This process is commonly suggested in amphibians. Figure 4, which represents the distribution of TSD and XY- and ZW-type GSD in families of lizards, clearly indicate that the acquisition of GSD with male heterogamety is phylogenetically more ancient in lizards than the ZW-type female heterogametic GSD, for example, in Dibamidae, Scincidae, the lineage of Pygopodidae and Sphaerodactylidae containing Phyllodactylidae and Gekkonidae, and also the lineage of Iguanidae containing Chamaeleonidae and Agamidae. In this paper, an association between ZW and TSD is only discussed based on Fig. 4, and the event of transition between XY and ZW is not considered in the phylogeny of lizards. The authors should make discussion on this point.

Response: We did not include discussion related to the events of transitions between XY and ZW in our MS because, similar issues have been discussed recently in two reviews (Janes et al. 2008 and Pokorna and Kratochvil, 2009. In addition, lizard phylogeny is far from being resolved; therefore, comments on transition at this stage would be highly speculative.

2. Pages 110, 119. The latest paper by Smith et al. (2009) has clearly shown that the dosage of the Z-linked *DMRT1* gene has a key role for avian testis differentiation. But I think that this work has not certified that *DMRT1* is the sex determining gene because of the lack of gain-of-function experiments. And also, there has been no evidence to deny the presence of the female dominant gene. The *WPKCI* and/or *FET1* genes are still strong candidates of the ovary-determining gene, suggesting that avian sex determination is regulated by the interaction between dosage of the Z-linked *DMRT1* gene and the female dominant gene. It seems to be one sided-discussion that the authors explain the sex determination system in female heterogametic lizards by the dosage mechanism as *DMRT1* in birds and the dominant mechanism for inhibition of the dosage system as *DM-W* in *X. laevis*.

Response: Our discussion is based on the published evidence and indeed our discussion would

have been more robust if results on the relevant experiments on DMRT1 in chicken are published. Moreover, there are no publications, which suggest that avian sex determination is an interaction between dosage of Z-linked and W-chromosomal gene. Therefore, without such data, our discussion is relevant and not one sided.

3. Page 119. Is there any confirmed data or evidence that *SRY* interacts with *SOX3* in the process of testis determination? As far as I can remember, there is no supporting evidence for the idea mentioned in Graves (1998).

Response: We reworded the sentence accordingly

Sex chromosome evolution in lizards: independent origins and rapid transitions

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Abstract

Reptiles epitomize the variability of reproductive and sex determining modes and mechanisms among amniotes. These modes include gonochorism (separate sexes) and parthenogenesis, oviparity, viviparity, and ovoviviparity, genotypic sex determination (GSD) with male (XX/XY) and female (ZZ/ZW) heterogamety and temperature dependent sex determination (TSD). Lizards (Order Squamata, Suborder Sauria) are particularly fascinating because the distribution of sex-determining mechanisms shows no clear phylogenetic segregation. This implies that there have been multiple transitions between TSD and GSD, and between XY and ZW sex chromosome systems. Approximately 1000 species of lizards have been karyotyped and among those, less than 200 species have sex chromosomes, yet they display remarkable diversity in morphology and degree of degeneration. The high diversity of sex chromosomes as well as the presence of species with TSD, imply multiple and independent origins of sex chromosomes, and suggest that the mechanisms of sex determination are extremely labile in lizards. In this paper, we review the current state of knowledge of sex chromosomes in lizards and the distribution of sex determining mechanisms and sex chromosome forms within and among families. We establish for the first time an association between the occurrence of female heterogamety and TSD within lizard families, and propose mechanisms by which female heterogamety and TSD may have co-evolved. We suggest that lizard sex determination may be much more the result of an interplay between sex chromosomes and temperature than previously thought such that sex determination mode is influenced by the nature of the heterogamety and as well as temperature sensitivity and the stage of sex chromosome degeneration.

Introduction

Sex chromosomes differ from autosomes in that the two members of the sex chromosome pair typically vary in morphology and gene content. They are highly specialized and appear to have evolved independently many times in vertebrates (for review see (Graves, 2008). Sex chromosomes are thought to evolve from an autosomal pair by the acquisition of a male or female-determining gene that defines a non-recombining region that is progressively extended, promoting degeneration of the sex-specific chromosome (Charlesworth, 1991; Muller, 1914; Ohno, 1967). This mode of sex determination is often referred to as chromosomal sex determination or genotypic sex determination (GSD).

GSD vertebrates typically have either a male heterogametic (XY male/XX female) or a female heterogametic (ZZ male/ZW female) sex chromosome system. The XX/XY sex chromosome system is conserved in therian mammals, and a ZZ/ZW pair is conserved within birds and within snakes (Graves, 2000; Matsuda et al., 2005; Ohno, 1967). The sex chromosome pair (X and Y, or Z and W) may differ only in a restricted region (even a single locus), as is expected given their autosomal origin, or can be highly differentiated as a result of progressive degeneration due to suppression of recombination. However, vertebrate XY and ZW sex chromosomes are not homologous, suggesting the independent evolution of sex chromosomes in different lineages from non-homologous ancestral autosomes (Fridolfsson et al., 1998; Nanda et al., 2002; Nanda et al., 2000). In some lineages, notably birds and mammals, sex chromosomes and sex determination are very stable. For example, all mammals have GSD with male heterogamety and sex is determined in therian mammals by the presence of a master sex determining gene (e.g. *SRY*), on the Y chromosome (Sinclair et al., 1990). All birds also have GSD but with female heterogamety and sex, at least in chicken, is determined by dosage of the gene *DMRT1* gene located on the Z chromosome (Smith et al., 2009).

Many reptiles have GSD but, unlike birds or mammals, sex can also be determined by incubation temperature (temperature-dependent sex determination or TSD) independent of specific genes or chromosomes (Bull, 1983; Charnier, 1966). In yet others, sex is determined by an interaction between environmental influences and genetic factors (Quinn et al., 2007; Radder et al., 2008). The widespread influence of temperature on sex determination may provide a viable state from which novel master sex genes and chromosomes in reptiles can evolve (Georges et al., 2009; Quinn, 2008) and influence directly the manner in which sex chromosomes are acquired and lost. The high diversity of sex determining mechanisms seen in

reptiles (e.g. XY, XXY, ZW, ZZW, TSD, genetic-environment interactions; Table, 1, Figure 1) is testament to the evolutionary lability of reptile sex determination.

There are close to 5000 lizard species (Uetz, 2009) and they exhibit an astonishing array of reproductive and sex determining modes including gonochorism (separate sexes) and parthenogenesis, oviparity, viviparity, and ovoviviparity, GSD and TSD, and male (XX/XY) and female (ZZ/ZW) heterogamety. They are particularly fascinating because the distribution of sex-determining mechanisms shows no clear phylogenetic segregation (Janzen and Phillips, 2006; Organ and Janes, 2008; Pokorná and Kratochvíl, 2009) (Table 1, Figure 1).

Lizards with GSD display remarkable diversity in sex chromosome differentiation, ranging from cryptic or homomorphic to highly differentiated (Figure 2). Much of this variation occurs within families, often among closely related species and even within the various races or populations of the same species. For example, the gekkonid lizard, *Gehyra purpurascens*, displays two Z chromosome and six W chromosome morphs, primarily as the result of centromeric inversions (Moritz, 1984) (Figure 2). Variation in the morphology of sex chromosomes among closely-related taxa, or populations of the one taxon, indicate that morphological evolution of sex chromosomes, and perhaps also sex determining mechanisms in lizards may occur relatively easily in comparison to mammals and birds.

There are substantial gaps in our knowledge of the distribution and characteristics of sex chromosomes within and among lizard families. There are data from all member species in only two families, one of which is monotypic and the other bitypic (Rhineuridae and Annielidae respectively), and for many of these, only one animal has been examined, thereby excluding an examination for sex chromosomes (Table 1). In addition, many lizards have sex microchromosomes that are difficult to identify using standard banding and staining techniques. Modern cytogenetic techniques, such as comparative genomic hybridization (CGH) that are more sensitive than G and C banding have enabled the characterization of sex chromosomes across a much wider range of species. This includes the identification of sex chromosomes that were cryptic because they involve microchromosomes or because they have undergone little morphological differentiation (Ezaz et al., 2005; Kawai et al., 2007; Traut et al., 1999).

Here, we review the current state of knowledge of sex chromosomes in lizards and the distribution of sex determining mechanisms and sex chromosomal forms within and among families. We establish for the first time an association between the occurrence of female heterogamety and TSD within lizard families, and propose mechanisms by which female

heterogamety and TSD may have co-evolved. We suggest that lizard sex determination may be much more the result of an interplay between sex chromosomes and temperature than previously thought such that sex determination mode is influenced by the nature of the heterogamety, temperature sensitivity and the stage of sex chromosome degeneration.

An astonishing variety of sex chromosomes in lizards

Karyotype information is available for at least one species from 29 of the 32 lizard families (Olmo and Signorino, 2005; Vidal and Hedges, 2009; Table 1). No species of Cadeidae, Lanthanotidae or Shinisauridae have been examined. Seven families (Iguanidae, Scincidae, Sphaerodactylidae, Pygopodidae, Dibamidae, Teiidae and Gymnophathalmidae) include species with male heterogamety (XX/XY) and six families (Bipedidae, Lacertidae, Varanidae, Chameleonidae, Agamidae and Phyllodactylidae) species with female heterogamety (ZZ/ZW). Only one family (Gekkonidae) includes species with both male and female heterogamety. No sex chromosomes have been detected in the remaining 15 families (Table 1).

At the level of species, approximately 913 have been karyotyped. Of these, sex chromosomes have been detected in only 181 species of which, about two thirds (115, 64%) have male heterogamety (XX/XY) and the remainder (66, 36%) female heterogamety (Table 1, Figure 1).

Although the majority of lizards with sex chromosomes have XY or ZW type systems, approximately 23% have multiple sex chromosomes (Figure 1). Multiple sex chromosome systems, thought to have evolved via autosome-sex chromosome fusions (King, 1977; Odierna et al., 2001; Olmo, 1986; Wright, 1973)(Leache and Sites, this issue), are common in XY, but not in ZW species. Only four (6%) of all female heterogametic species have multiple sex chromosomes systems ($Z_1Z_1Z_2Z_2$ male and Z_1Z_2W female, Table 1, Figure 1, Figure 2) and these are confined to the Family Lacertidae (3 species of *Lacerta* and one species of *Podacris* (Olmo and Signorino, 2005) (Table 1). In contrast 37 (32%) of the 115 species with XY sex chromosomes have multiple sex chromosomes ($X_1X_1X_2X_2$ female and X_1X_2Y male), most of which occur in Iguanidae (33/37, Table 1, Figure 2,).

In addition, both simple and multiple sex chromosomes have evolved within closely related species (e.g. *Anolis*) and even different populations of the same species have been found to include multiple and simple sex chromosomes systems. Intra-specific variation involving multiple XY sex chromosomes systems occurs in populations of *Scincella lateralis* (XY/XXY, Figure 2) (Wright, 1973) and *Sceloporus clarkii* (Leache and Sites, this issue), while multiple ZW systems occur in *Lacerta vivipera* (ZW/ZZW)(Odierna et al., 2001).

A number of mechanisms have been proposed to explain the formation of heteromorphic sex chromosomes in lizards (Bickham, 1984; Cole et al., 1967; King, 1981; Olmo, 1986; Olmo et al., 1987). Most of these are derived from observation through classical cytogenetic analysis (mainly differential staining and banding) and include centromeric inversion (peri or para), centric fusion, heterochromatinization followed by amplification of tandem repeats, heterochromatinization followed by degeneration, and autosome-sex chromosome translocation. Rather than degeneration, as seen in the sex chromosomes of mammals and most birds and some snakes, the sex-specific chromosomes (Y or W) in many lizards are larger than their homologues, usually as a result of the tandem amplification of repetitive elements. For example, in Varanidae, the W chromosome is substantially larger than the Z chromosome in all four species with sex chromosomes, and is thought to have expanded by tandem amplification of heterochromatin (King, 1977; Figure 2).

Like birds, turtles and snakes, most lizards have a karyotype composed of macrochromosomes (ranging from 2n = 10-46) and microchromosomes (ranging from 2n = 0-26)(Olmo and Signorino, 2005). Microchromosomes have been found to be gene rich in birds with 2-3 times the number of genes than contained in macrochromosomes (Smith et al., 2000) and to have higher recombination rates (Rodionov et al., 1992). In addition, microchromosomes are GC, and CpG-rich and contain few repetitive elements (Hillier et al., 2004) and therefore, are likely to be important for generating genetic variation (Organ et al., 2008). In contrast, very little (primarily from classical cytogenetics) is known about the origin, evolution and genomics of microchromosomes in lizards (Olmo, 2008).

Importantly, in some species of lizards, sex chromosomes have been found to be microchromosomes (Bull, 1983; Ezaz et al., 2005; Ezaz et al., 2009a; Gorman, 1973; Gorman and Atkins, 1966). Sex microchromosomes have been found to be in most species of Iguanidae with multiple sex chromosomes, in all four species so far examined in Varanidae and four out of the five species examined in Agamidae (Ezaz et al., 2005; Ezaz et al., 2009a; Gorman and Aitkins, 1968; Gorman and Atkins, 1966; King and King, 1975; King et al., 1982). The patterns of differentiation of these sex microchromosomes are highly variable within and among groups (Figure 2), and have evolved primarily via the accumulation and amplification of heterochromatin.

Sex microchromsomes appear to be highly labile in at least one family of lizards, with agamids exhibiting a substantial array of forms among closely related species. Four out of five agamid lizards whose sex chromosomes are known (*Pogona vitticeps*, *P. barbata*, *Amphibolurus nobbi*

and *Ctenophorus fordi*) have ZW sex microchromosomes that are among the smallest in the complement (Ezaz et al., 2005; Ezaz et al., 2009a; Quinn et al., 2009a). In this group, the W chromosomes are highly to moderately heterochromatic whereas, the Z chromosomes are euchromatic and can be detected only by mapping sex chromosome specific DNA sequences (Ezaz et al., 2005; Ezaz et al., 2009a) or by mapping sex chromosome specific BAC clones (Ezaz unpubl.). Both the Z and W chromosomes are DAPI faint in all four species, suggesting they comprise GC rich sequences. The C-banding pattern on the W chromosomes are also variable among the four dragon species, ranging from fully heterochromatic in *P. barbata* and *P. vitticeps*, to highly heterochromatic in *A. nobbi* and dot-like in *C. fordi* (Ezaz et al., 2005; Ezaz et al., 2009a). These patterns of heterochromatic variability suggest various stages of sex chromosome differentiation within closely related species.

Cryptic or homomorphic sex chromosomes are often considered to be the norm in fish, amphibians and reptiles. While this may be true for fish and amphibians where GSD is the prevalent mode of sex determination, the frequency of homomorphic sex chromosomes in reptiles is under-reported because incubation experiments have been needed to distinguish GSD species with homomorphic sex chromosomes from TSD species that lack sex chromosomes. These data are available for relatively few lizard species (Table 1). Nevertheless, homomorphic sex chromosomes are certainly likely to be common in GSD lizards, as they are in fish and amphibians. High turnover of sex chromosomes, where new master sex determining genes arise on autosomes to regularly reset the progressive deterioration of the Y or W (Schartl, 2004; Volff et al., 2007), and generation of novel Y and W haplotypes by temperature induced sex reversal (Perrin, 2009), have been suggested as possible causes. Regular transitions between ZW dosage and XY dominance (or vice versa) involving the same master sex gene and associated chromosomes (Quinn, 2008) may also play a role.

The conclusion we draw from the studies reviewed above is that the lizard genome is dynamic at the level of sex chromosome organization. There is significant variation in the mode of sex determination and variation in the degree of heteromorphism with little regard for phylogenetic relationship or distance. Such variation occurs between families, species and even populations of the one species.

Multiple origins of sex chromosome in lizards

The extreme variation in sex determining mechanisms within lizards is not typical of all reptile groups. Sex chromosomes are conserved within several groups. For example, chromosome 4 is the Z chromosome in all snakes studied to date (Becak et al., 1963; Becak et al., 1964; Ohno,

1967; Solari, 1993), whereas, chromosome pairs 4 or 5 are Z chromosomes in most birds (Ohno, 1967; Ohno et al., 1964; Solari, 1993; Suzuki, 1930). Chromosome painting attested to the homology of the Z chromosome, even between the most distantly related birds (Shetty et al., 1999), and the gene content of the bird Z chromosome is also conserved (Fridolfsson et al., 1998; Stiglec et al., 2007). So is the gene content of the snake Z between three distantly related species (*Python molurus*, *Elaphe quadrivirgata*, *Trimeresurus flavoviridis*; Matsubara et al., 2006). In each lineage, the W chromosome shows homology to the Z, but is degraded by different degrees (Bull, 1980; Bull, 1983; Ohno, 1967).

The similar sizes of the snake and bird Z chromosomes were initially thought to reflect ancient homology (Ohno, 1969). However, gene mapping showed that the bird and snake Z chromosomes are non-homologous; the bird Z is completely contained within the short arm of snake chromosome 2, and the snake Z corresponds to the bird chromosomes 2 and 27 (Matsubara et al., 2006; Matsuda et al., 2005). Several recent studies using comparative gene mapping in snakes, a species of turtle (*Pelodiscus sinensis*), a species of gecko (*Gekko hokouensis*) and a species of dragon lizard (*Pogona vitticeps*) have shown that sex chromosomes, particularly ZW pairs, are not homologous between reptile groups (Ezaz et al., 2009b; Kawagoshi et al., 2009; Kawai et al., 2009; Kawai et al., 2007; Matsubara et al., 2006; Matsuda et al., 2005). This is consistent with the idea that sex chromosomes evolved many times independently in reptiles (Figure 3).

Comparative mapping between snakes, birds, the gecko *G. hokouensis* and the agamid lizard *P. vitticeps* confirms the independent origin of their sex chromosomes. Four genes (*ATP5A1, GHR, DMRT1, CHD1*) that are sex linked in the gecko *G. hokouensis* were found to be autosomal in the dragon lizard *P. vitticeps* (Ezaz et al., 2009b; Kawai et al., 2009). Similarly, five snake sex linked genes (*WAC, KLF6, TAX1BP1, RAB5A* and *CTNNB1*), lie on chromosome 6, and five chicken Z chromosome-borne genes are on chromosome 2 in *P. vitticeps* (Figure 3). Thus, the ZW pair of agamid lizard *P. vitticeps* is not homologous either to those of birds or snakes or the gecko *G. hokouensis* (Figure 3), (Ezaz et al., 2009b). This suggests independent origins of sex chromosomes not only between two lizard species, but also among species of lizards snakes and a turtle. The independent evolution of sex chromosomes is likely to be more common in lizards as more species are subjected to comparative study.

Lizards also show much variation within groups although, with only six functional sex linked genes, and four sex chromosome or sex-linked markers mapped or tested in only five species of lizards (Ezaz et al., 2009b; Quinn et al., 2009a; Quinn et al., 2009b), the degree of variation is

debatable. W-specific markers from the Komodo dragon were found to be sex linked in the Australian varanid *V. rosenbergi* (W. Smith, pers. comm), suggesting the conservation of sex chromosome sequences within Varanidae. ZW-linked AFLP markers isolated from the dragon lizard, *P. vitticeps* were found by PCR analysis to be sex-linked in a closely related species, *P. barbata*. However, they were also found to be autosomal in several other species tested (Quinn et al., 2009b), suggesting the independent evolution of sex-chromosome specific sequences within Australian agamids. Also, in a preliminary study, a sex-linked AFLP marker isolated *from Bassaina duperryei* was found not to be sex-linked in a species of Tasmanain skink *Novoscincus ocellatues* (Ezaz unpub.), suggesting independent origin of sex specific sequences in skinks despite their morphologically conserved sex chromosomes (Donnellan, 1985; Hutchinson and Donnellan, 1992).

Another recent study on the comparative mapping of *P. vitticeps* sex chromosome specific marker has provided evidence for the rapid evolution of non-homologous ZW sex chromosomes within Australian dragon lizards (Ezaz et al., 2009a). Cross-species chromosome painting using a *P. vitticeps* sex chromosomal marker (Quinn et al., 2009b) revealed that the ZW sex microchromosomes of three Australian species (*P. vitticeps*, *P. barbata* and *A. nobbi*) were homologous, but that those of a fourth species, *C. fordi,* were not. This suggests at least two independent origins of ZW sex microchromosome systems in Australian agamids (Ezaz et al., 2009a). No comparative data on the sex chromosomes in Iguanidae and Varanidae are available, so homology between the sex chromosomes of these three lizard groups is unknown.

These demonstrations of variation in lizard sex chromosomes, as well as the presence of species with temperature dependent sex determination, imply multiple and independent origins of sex chromosomes, and suggest that the mechanisms of sex determination are extremely labile in this group. This lability may indicate frequent transitions between modes (TSD and GSD) and mechanisms (XY and ZW) of sex determination in lizards through the evolution of novel sex chromosomes, perhaps via the acquisition of novel genes. The activation of unlinked sex determining genes on autosomes could also be a possible mechanism for the independent origin of sex chromosomes in different lizard lineages, as proposed for the evolution of non-homologous sex chromosomes in salmonid fishes (Woram et al., 2003).

An ancient reptile ZW pair?

Against this variation, the conservation of the ZW pair between birds and the gecko, *G. hokouensis* is remarkable, suggesting these two distantly-related taxa have conserved synteny of six functional genes over more than 285 MYA (Kawai et al., 2009; Rest et al., 2003). A

parsimonious view of this finding is that chicken and gecko *G. hokouensis* retain the primitive condition of their common ancestor, with the remaining squamate clade (Vidal and Hedges, 2009) having more recently derived states, albeit, independently derived (Ezaz et al., 2009b). However, in the context of the very great evolutionary lability of sex chromosomes in lizards, an alternate explanation presents itself. The master sex determining genes of chicken and gecko may well be different but by chance have come to reside on homologous chromosomal regions, either by the chance capture of sex determination by genes in a syntenic region common to the bird-gecko ancestor, or through translocation. Translocation is more likely to happen in lizards, because of the apparent lack of morphologically differentiated sex chromosomes which is likely to represent nascent sex chromosomes, and such nascent sex chromosomes are known to accumulate transposable elements and have been reported in a wide range of plants and animals (Adams MD, 2000; Charlesworth et al., 2005; Kejnovsky et al., 2008; Matsunaga, 2009; Skaletsky et al., 2003).

Support for convergence in the chromosomal location of the master sex determining gene comes from the observation that it is unlikely that birds and gecko have the same master sex determining genes. The male determining gene *DMRT1* in chicken is present only on the Z chromosome and sex is determined by dosage of the *DMRT1* gene product, two copies produce males, whereas one copy produces females (Smith et al., 2009). In *G. hokouensis*, this gene is present in both Z and W chromosomes (Kawai et al., 2009), making it unlikely to be sex determining in this species unless a paralogue of *DMRT1* is present and functions as a dominant master gene (as found in *Xenopus leavis*; *DMW*; Yoshimoto et al., 2008), which is likely to function as a suppressor of autosomal *DMRT1* dosage. Moreover, closely related species of gecko have both XY and ZW sex chromosome systems as well as TSD, and possibly sex chromosome-temperature interactions (e.g. *Gekko japonicus*; Gamble, 2009; Tokunaga, 1985), indicating rapid transitions between modes of sex chromosomes as well as modes of sex determination (for review see Gamble, 2009). The emergence of a novel sex determining gene in gecko in the 285 million years since their divergence from birds, and their chance residence on homologous chromosomes, would seem at least an equally plausible explanation for the homology of the sex chromosomes of these taxa, given the lability of sex chromosomes and mechanisms in other lizards. The matter is unlikely to be resolved until the genetic mechanisms of their sex determination become better known and without a comprehensive genomic analysis across the squamate phylogeny (e.g. geckos, skinks, varanids, lacertids, agamids).

Whatever the mechanism of sex determination in the gecko, the homology between the gecko and bird sex chromosomes suggests that a bird-like ZW could be ancestral for reptiles. The

homology between the bird ZW and the XY complex of the platypus (Veyrunes et al., 2008) suggests that this homology might extend back to a common amniote ancestor that lived 310 million years ago (Graves, 2008). Alternatively, if ZW is the ancestral state, some of the fundamental genetic machinery of a ZW-driven sex determination and differentiation network may have been retained to varying degrees in different reptile lineages, leading to a predisposition to evolve similar sex determining mechanisms. Thus similar sex chromosome systems may have independent origins, but are constrained in some way by their phylogenetic history.

An association between TSD and female heterogamety in lizards

In temperature dependent sex determination (TSD), males and females are produced differentially according to the incubation temperatures experienced by the developing embryos. Although TSD was first described in an agamid lizard, *Agama agama* (Charnier, 1966), it is more frequent in non-squamate reptiles (in possibly all crocodilians, many turtles, tuatara) than lizards. Since Charnier's discovery, TSD has been identified unequivocally in 37 species from six families, most of which are from the Agamidae (13/37) and Gekkonidae (8/37, Sup Table 1, Figure 1) families. In addition, both GSD (4 species) and TSD (5 species, Sup Table 1) have been detected in the family Eublepharidae, although no sex chromosomes are known.

Evidence of TSD has been reported in another seven species from the families Lacertidae (one species), Scincidae (four species), Anguidae (one species) and Varanidae (one species) (see Harlow, 2004; Pokorná and Kratochvíl, 2009). However, these data are equivocal mainly because of small sample size as well as the nature and design of the incubation experiments and sex identification techniques employed (see Harlow 2004, Pokorna and Kratochvil 2009), so we did not include those species as having TSD in this review.

The classical view of transitions between GSD and TSD has sex captured by a temperature sensitive element, the production of lethal or suboptimal YY or WW individuals, and the loss of these chromosomal elements from the genome through natural selection or drift (Bull, 1980; Bull, 1983). GSD arises from TSD through the capture of sex determination by novel genes which come to influence sex and the formation of sex chromosomes (Bull, 1980; Bull, 1983; Charlesworth, 1991). However, there is emerging evidence of interactions between GSD and TSD both in forms regarded as GSD (*Bassiana duperreyi*, *Pogona vitticeps*) (Quinn et al., 2007; Radder et al., 2008) and forms regarded as TSD (*Amphibolurus muricatus* and *Agama impalearis* have 50:50 sex ratios at intermediate temperatures,(El Mouden et al., 2001; Harlow

and Taylor, 2000) and that sex determination should be regarded more as a continuum of states than a dichotomy of GSD and TSD (Sarre et al., 2004).

Mapping the distribution of TSD and GSD across the lizard phylogeny shows an almost haphazard distribution of the traits. However, one trend demands some attention: an association between the occurrence of TSD and the occurrence of female heterogamety within families (Figure 4). This suggests that ZW sex chromosomes evolved only in those families in which species with TSD occur. An exception is the Gekkonidae, which contains species with XY, ZW or TSD (Figure 4). Is this association an artifact, arising from limited data on the mode of sex determination in majority of the lizard lineages? Or does the pattern arise from fundamental underlying processes we do not yet fully understand?

An association might suggest that ZW systems are more prone to coming under thermal influence, or perhaps female heterogamety is in some way more compatible with selection for TSD when thermal tendencies first arise. Alternatively, it might mean that ZW systems are more compatible with the retention of an underlying genetic predisposition (cryptic sex-linked ZW chromosomes) in TSD species (Quinn 2008). It has also been suggested that ZW systems in lizards act via dosage mechanisms that are more likely to evolve TSD than those systems that act via male dominance (Quinn et al. 2007), and it is gene dosage systems that are more susceptible to temperature influence than dominant gene systems.

These questions arise from an apparent association between ZW systems and TSD in the lizard phylogeny but our knowledge of the mechanisms of sex determination in lizards, and in particular, the interaction between genotype and temperature in determining sex, is as yet too rudimentary to take the ideas beyond speculation. The fundamental differences to the molecular mechanisms of sex differentiation between female homogametic and female heterogametic system is not fully known. There is evidence that both dominant and dosage mechanisms can determine sex in ZW systems (e.g. *DMRT1* dosage in birds, Smith et al. 2009; DMW dominance in *Xenopus leavis*, Yoshimoto et al. 2008), however, no XY dosage systems have been observed in any vertebrate. It is likely that among female heterogametic lizards, some will determine sex via dosage mechanisms as in birds, while others via dominant mechanisms, as in *X. leavis*. The dominance of a master sex determining gene could be achieved by regulation of one or more of the influential genes in the sex differentiation pathway, in the way *SRY* interacts with *SOX3* or *SOX9* as hypothesized previously in mammals (Graves, 1998). Alternatively, it could be achieved by inhibition of what was formerly a dosage system, in which the dosage is inhibited or knocked down by members of same gene family as

is suspected in *X. leavis* (likely *DMW* inhibition of *DMRT1*). Thus, regardless of heterogamety, sex in many lizard clades may be determined by gene dosage or gene dosage captured by a "dominant" master sex gene and so generally predisposed to capture of the genetic pathway of sex differentiation by exogenous cues, such as temperature.

Further studies, particularly in gekkonid and agamid lizards, could reveal the nature of such co-evolution of ZW sex chromosomes and TSD. For example, Gekkonidae is the only family where XY, ZW and TSD exist (Gamble 2009). If XY and ZW sex chromosomes are found to be homologous (e.g. *Rana rugosa,* Ogata et al., 2003), and if closely related species or populations have TSD (e.g. *Gekko japonicus*, *G. gecko*, *G. hokouensis*) then identifying sex determining genes in these species and comparative analyses of gonad differentiation pathways in XY, ZW, TSD populations (species) of geckos would reveal the true nature of co-evolution of TSD and ZW sex chromosomes and mechanisms of transitions between modes. Evidence for a relationship between TSD and ZW can also be sought in species from Agamidae, particularly Australian dragon lizards, in which these features have been reliably identified (Harlow 2004, Ezaz et al. 2005, Quinn et al. 2007, 2009, Ezaz et al. 2009a,b) and genomics resources are already available.

Conclusions

Lizards occupy a key evolutionary position in the amniote phylogeny, so understanding the origin, evolution and mechanisms of sex determination and sex chromosomes will help us to better understand the origin and evolution of sex chromosomes in other vertebrates including mammals. In particular, such studies will resolve the debate about the ancestry of sex determination, TSD, or GSD, XY or ZW, but they will also unravel how GSD and TSD co-exist and how transitions between heterogamety occur.

Fewer than 200 species of lizards have identifiable sex chromosomes, yet they display remarkable diversity in morphology and extent of degeneration. The high diversity of sex chromosomes as well as the presence of species with TSD, imply multiple and independent origins of sex chromosomes, and suggest that the mechanisms of sex determination are extremely labile in lizards. This apparent lability is likely to have a significant role in rapid transitions among modes and mechanisms perhaps via female heterogamety and TSD. However, little is known about the genomics of sex chromosomes and their relationship with the plasticity of sex determination in lizards.

How sex chromosomes and temperature interact to determine sex in many lizard species is not known, although theoretical models have been proposed (Bull 1983, Geroges et al. 2009, Quinn 2008). Thermal influence on sex reversal in two lizard species with highly heteromorphic sex chromosomes (Radder et al. 2008, Quinn et al. 2007) is an indication of the propensity of sex determination to be sensitive to temperature among lizards. It is quite likely that sex chromosomes will be found in lizard species that have long been regarded as classical TSD species. Therefore, interactions between sex chromosomes and temperature may be more intrinsic in lizards than previously thought, and may depend on the extent of differentiation of sex chromosomes as well as heterogamety. The apparent co-occurrence of TSD and female heterogamety may provide such a clue and perhaps represent a transitional phase between alternate modes and mechanisms.

Therefore, lizard sex determination may be much more the result of an interplay between sex chromosomes and temperature than previously thought, such that the sex determination mode is influenced by the nature of the heterogamety, temperature sensitivity and the stage of sex chromosome degeneration. Future comparative genomic analysis of sex chromosomes among closely related lizard species would appear to be one of the most important steps to understanding the origin, evolution and transitions of sex chromosomes and sex determinations. Recent advances in molecular genetics, cytogenetics and sequencing technologies promise to advance our knowledge substantially in the near future.

Acknowledgements

We thank Ettore Olmo for the opportunity to contribute to this special issue. Many thanks to T. Gamble for discussion about sex chromosomes and sex determination in lizards and reptiles. This work is supported by the Australian Research Council Discovery Grant (DP0881196) awarded to S.S., A.G. and Scott V. Edwards.

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Tables and figures

Table 1. Current knowledge about the occurrence of sex chromosomes and TSD among lizard families. Data compiled from various sources including Olmo and Signorino 2005, Ezaz et al. 2005 and 2009a., Yonenaga-Yassuda, 1999, 2005, Donnellan 1989, Zeng et al. 2009, Kawai et al. 2009, Andrews 2005, Harlow 2004, Pokorna and Kratochvil 2009, Gamble 2009. TSD: temperature dependent sex determination, NKD: number of species karyotyped, SCH: Species with sex chromosomes, TNS: total number of species, PKD: proportion karyotyped, PSC: proportion with sex chromosomes. See supplementary reference list for citations.

	XY	XXY	ZW	ZZW	Cryptic	TSD	NKD	SCH	TNS	PKD	PSC
Amphisbaenidae	0	0	0	0	0	0	26	0	159	16	0
Trogonophidae	0	0	0	0	0	0	2	0	6	33	0
Bipedidae	0	0	1	0	0	0	3	1	3	100	33
Blanidae	0	0	0	0	0	0	2	0	4	50	0
Cadeidae	0	0	0	0	0	0	0	0	1	0	0
Rhineuridae	0	0	0	0	0	0	1	0	1	100	0
Lacertidae	0	0	39	4	0	0	104	43	225	46	41
Teiidae	2	0	0	0	0	0	62	2	83	75	3
Gymnophthalmidae	5	2	0	0	0	0	22	7	175	13	32
Anguidae	0	0	0	0	0	0	1	0	16	6	0
Annielidae	0	0	0	0	0	0	2	0	2	100	0
Diploglossidae	0	0	0	0	0	0	6	0	50	12	0
Helodermatidae	0	0	0	0	0	0	1	0	2	50	0
Xenosauridae	0	0	0	0	0	0	1	0	6	17	0
Lanthanotidae	0	0	0	0	0	0	0	0	1	0	0
Varanidae	0	0	4	0	0	0	23	4	68	34	17
Shinisauridae	0	0	0	0	0	0	0	0	1	0	0
Iguanidae	36	33	0	0	0	0	249	69	700	36	28
Chamaeleonidae	0	0	1	0	1		50	1	178	28	2
Agamidae	0	0	5	0	9	13	93	5	380	24	5
Scincidae	30	1	0	0	0	0	118	31	1200	10	26
Xantusiidae	0	0	0	0	0	0	12	0	29	41	0
Gerrhosauridae	0	0	0	0	0	0	12	0	34	35	0
Cordylidae	0	0	0	0	0	0	11	0	55	20	0
Sphaerodactylidae	1	0	0	0	0	0	5	1	196	3	20
Gekkonidae	2	0	11	0	0	8	75	13	794	9	17
Phyllodactylidae	0	0	1	0	0	6	7	1	109	6	14
Eublepharidae	0	0	0	0	4	5	9	0	29	31	0
Diplodactylidae	0	0	0	0	0	5	4	0	25	16	0
Carphodactylidae	0	0	0	0	0	0	6	0	28	21	0
Pygopodidae	1	1	0	0	0	0	5	2	40	13	40
Dibamidae	1	0	0	0	0	0	1	1	21	5	100

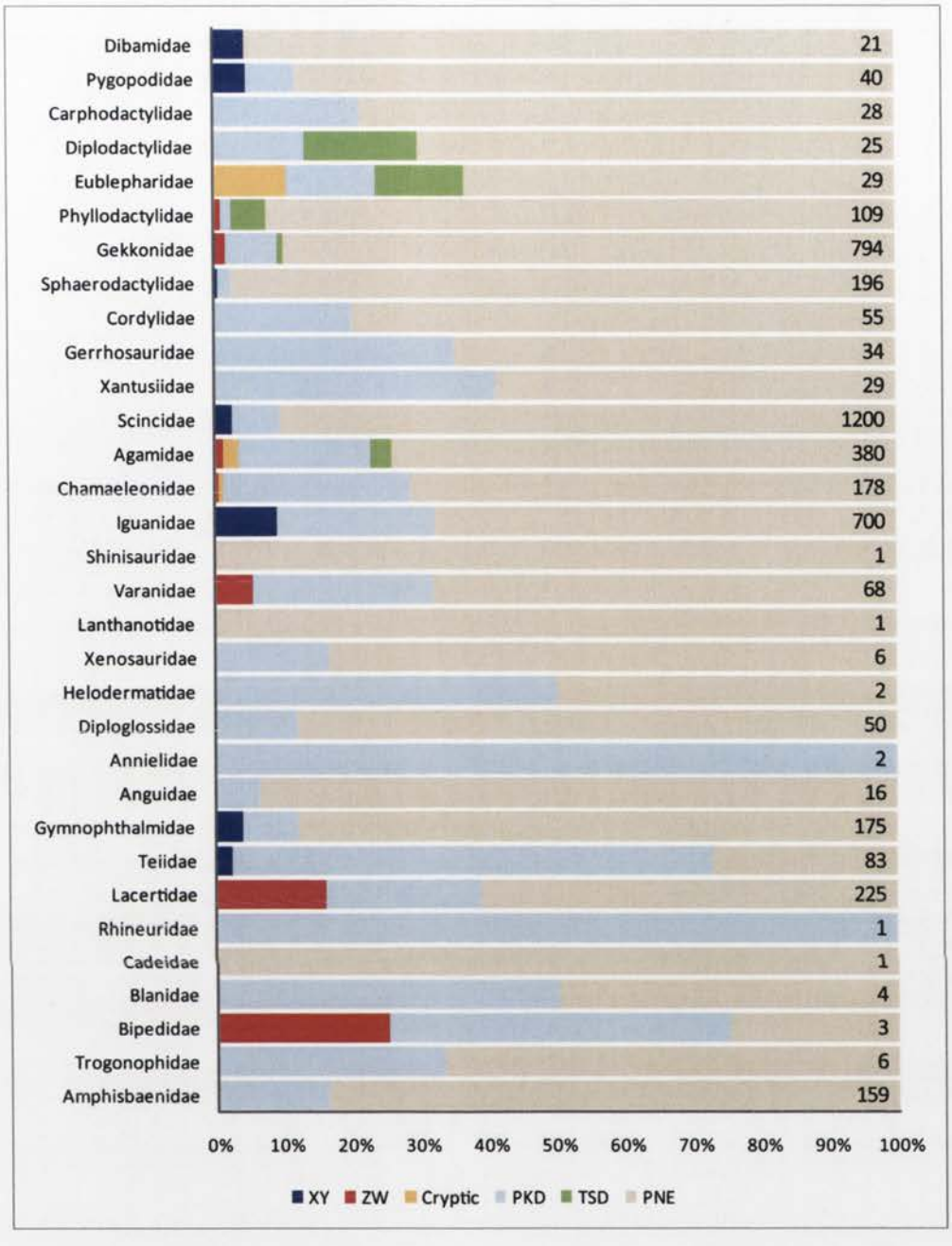

Figure 1. Schematic representation showing current status of occurrence and distributions of sex chromosome systems and sex determining modes among lizard families. Phylogeny is based on Vidal and Hedges (Vidal and Hedges, 2009). Numbers indicate total number of species (see Table 1 for detail). Data compiled from various sources (see supplementary reference list) including (Andrews, 2005; Donnellan, 1985; Ezaz et al., 2005; Ezaz et al., 2009a; Harlow, 2004; Kawai et al., 2009; Olmo and Signorino, 2005; Pokorná and Kratochvíl, 2009; Yonenaga-Yassuda and Rodrigues, 1999; Yonenaga-Yassuda et al., 2005). PKD: proportions karyotyped, TSD: temperature dependent sex determination, PNE: proportions not examined.

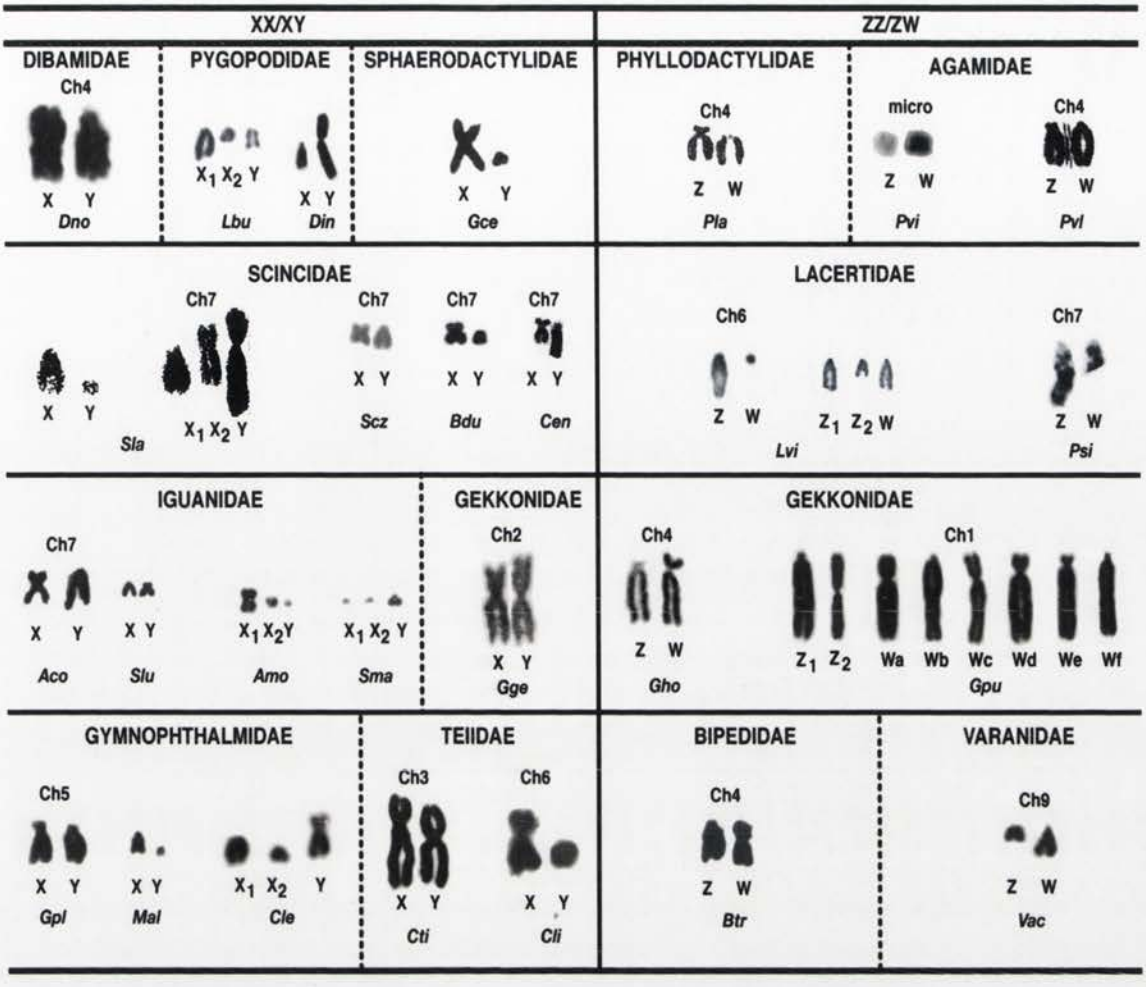

Figure 2. A snapshot of morphological diversity of sex chromosomes in lizard. *Aco*: *Anolis conspersus* (Gorman and Aitkins, 1968), *Amo: Anolis monesis* (Gorman and Stamm, 1975), *Bdu: Bassiana duperreyi* (Shine et al., 2002), *Btr: Bipes tridactylus* (Cole and Gans, 1987), *Cen: Claireascincus entrecasteauxii* (Hutchinson and Donnellan, 1992), *Cle: Calyptomatus leiolepis* (Yonenaga-Yassuda et al., 2005), *Cli: Cnemidophorus littoralis* (Peccinini-Seale et al., 2004), *Cti: Cnemidophorus tigris* (Cole et al., 1969), *Din: Delma inornata* (King, 1990), *Dno: Dibamus novaeguineae* (Cole and Gans, 1997), *Gce: Gonatodes ceciliae* (McBee et al., 1987), *Gge: Gekko gecko* (Solleder and Schmid, 1984), *Gho: Gekko hokouensis* (Kawai et al., 2009), *Gpl: Gymnophthalmus pleei* (Cole et al., 1990), *Gpu: Gehyra purpurascens* (Moritz, 1984), *Lbu: Lialis burtonis* (Gorman and Gress, 1970), *Lvi: Lacerta vivipera* (Odierna et al., 2001), *Mal: Micrablepharus allicolus* (Yonenaga-Yassuda and Rodrigues, 1999), *Pla: Phyllodactylus lanei* (King, 1981), *Psi: Podarchis sicula* (Odierna et al., 1993), *Pvi: Pogona vitticeps* (Ezaz et al., 2005), *Pvl: Phrynocephalus vlangalii* (Zeng et al., 1997), *Scz: Saproscincus czechurai* (Donnellan, 1991), *Sla: Scincella lateralis* (Wright, 1973), *Slu: Sceloporus lundelli* (Cole, 1970), *Sma: Sceloporus maculosus* (Cole, 1971), *Vca:Varanus acanthurus* (King et al., 1982). For citations see Supplementary reference list.

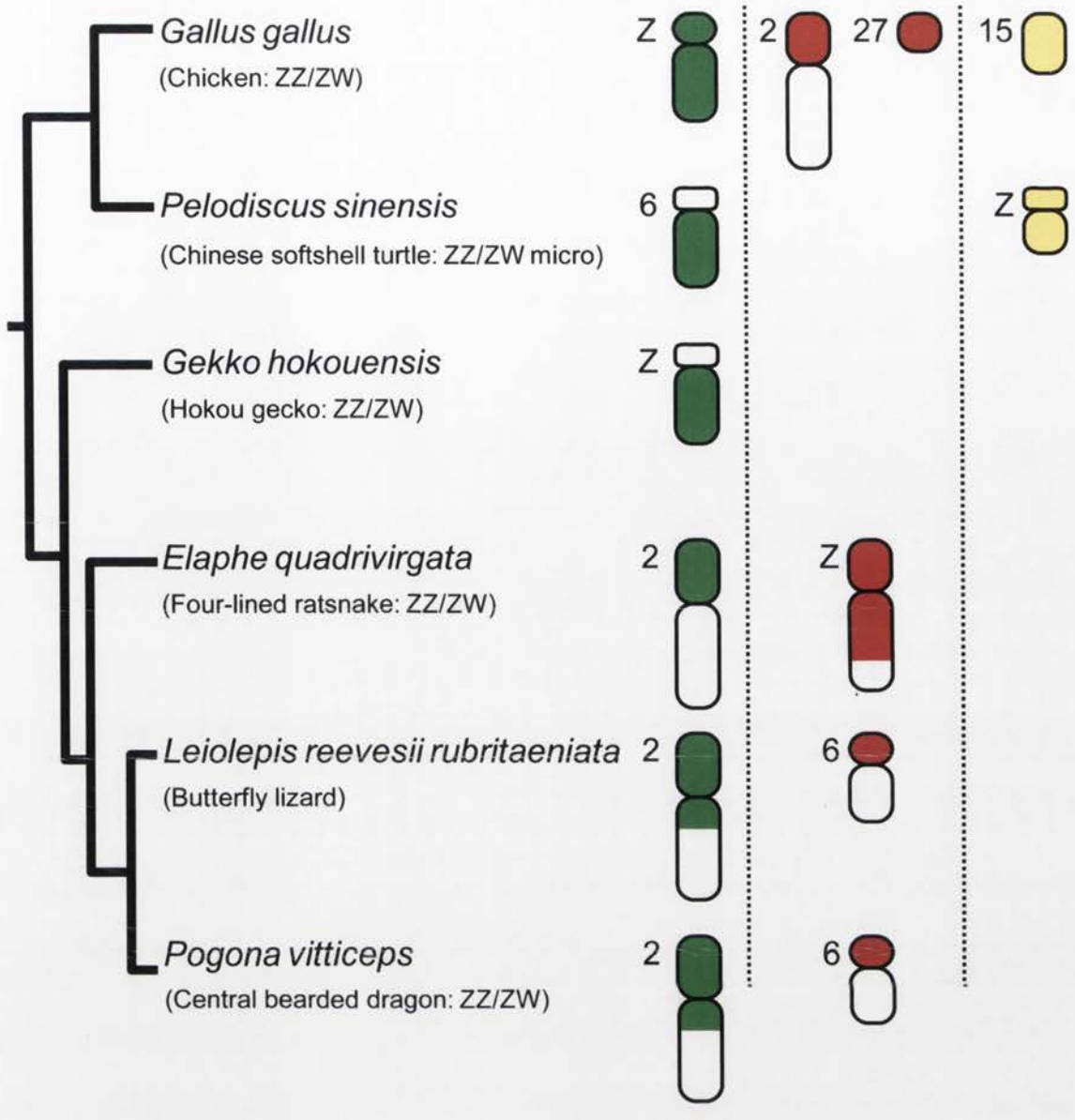

Figure 3. Schematic representation showing the non-homology and multiple and independent origins of ZZ/ZW sex chromosome systems in reptiles based on reciprocal mapping of sex chromosomal genes from chicken, snakes and turtle. Data are summarized after (Ezaz et al., 2009b; Ezaz et al., 2009a; Kawagoshi et al., 2009; Kawai et al., 2009; Kawai et al., 2007; Matsubara et al., 2006; Matsuda et al., 2005; Srikulnath et al., 2009), and http://www.ensembl.org/index.html. For citations see Supplementary reference list.

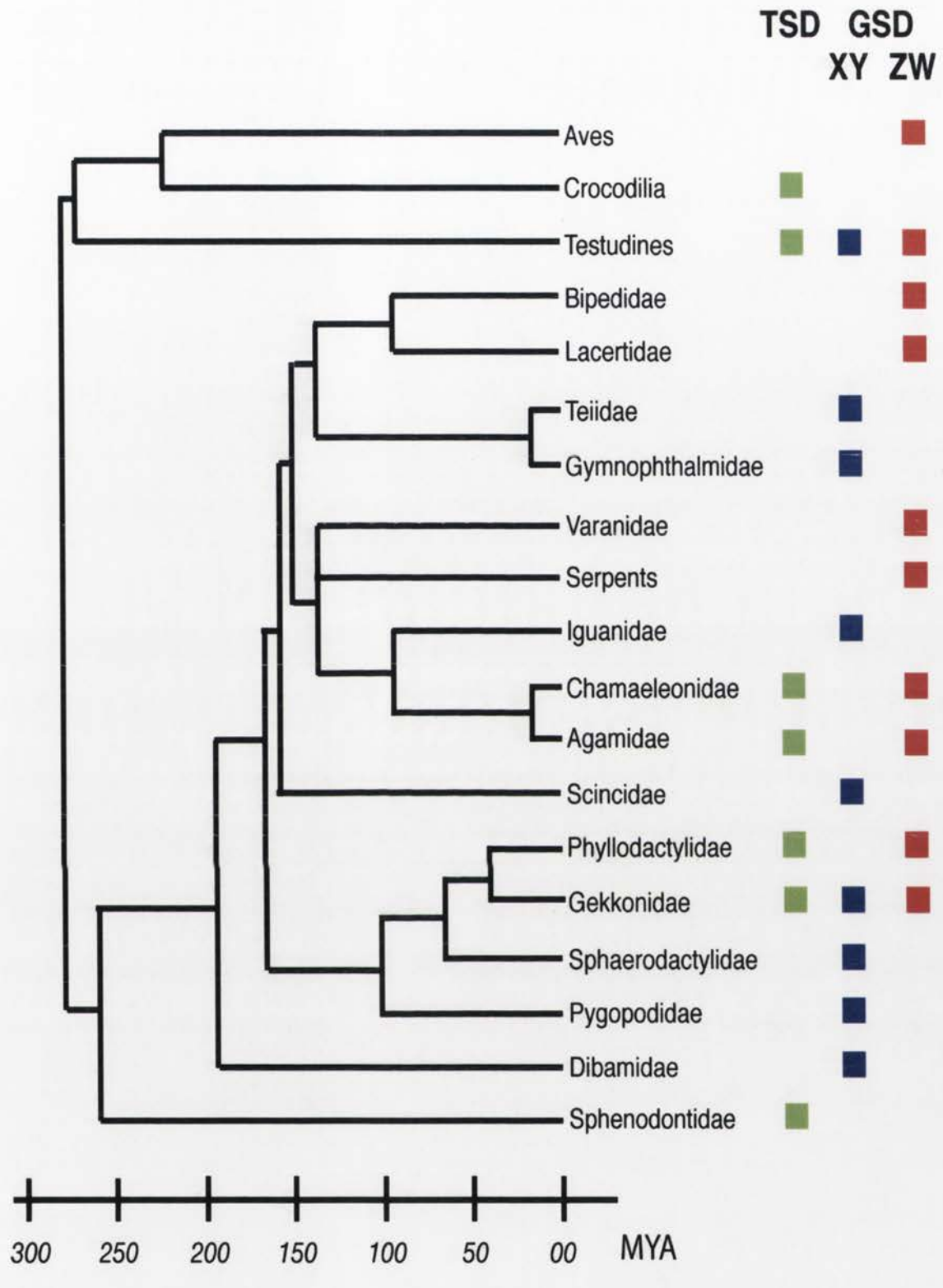

Figure 4. Phylogeny of reptiles (pruned phylogenetic tree modified after Vidal and Hedges, 2009) showing apparent association between TSD (temperature dependent sex determination) and female heterogamety in lizards, suggesting frequent transitions between modes and mechanisms of sex determination in lizards via TSD and female heterogamety.

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Current status of paper (circle as appropriate):

Planned/In Preparation/Submitted/Under Revision/Accepted/ Published

Date paper accepted for publication or anticipated date of publication:

January 2009

Name of journal:

Chromosome Research **17**:91-98

Extent to which research is your own:

I collected samples, performed laboratory work with my supervisor (T. Ezaz), performed analyses and interpreted results. I presented this work at a local conference, winning the student prize.

Your contribution to writing the paper:

I contributed to and edited several drafts of the manuscript and prepared figures.

If paper not yet accepted, has the paper been rejected by any journals:

N/A

Comments:

Chromosome Research (2009) 17:91–98
DOI 10.1007/s10577-008-9019-5

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

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Received: 23 October 2008 / Revised and Accepted: 14 November 2008 / Published online: 27 January 2009

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

Responsible Editor: Herbert Macgregor.

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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

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

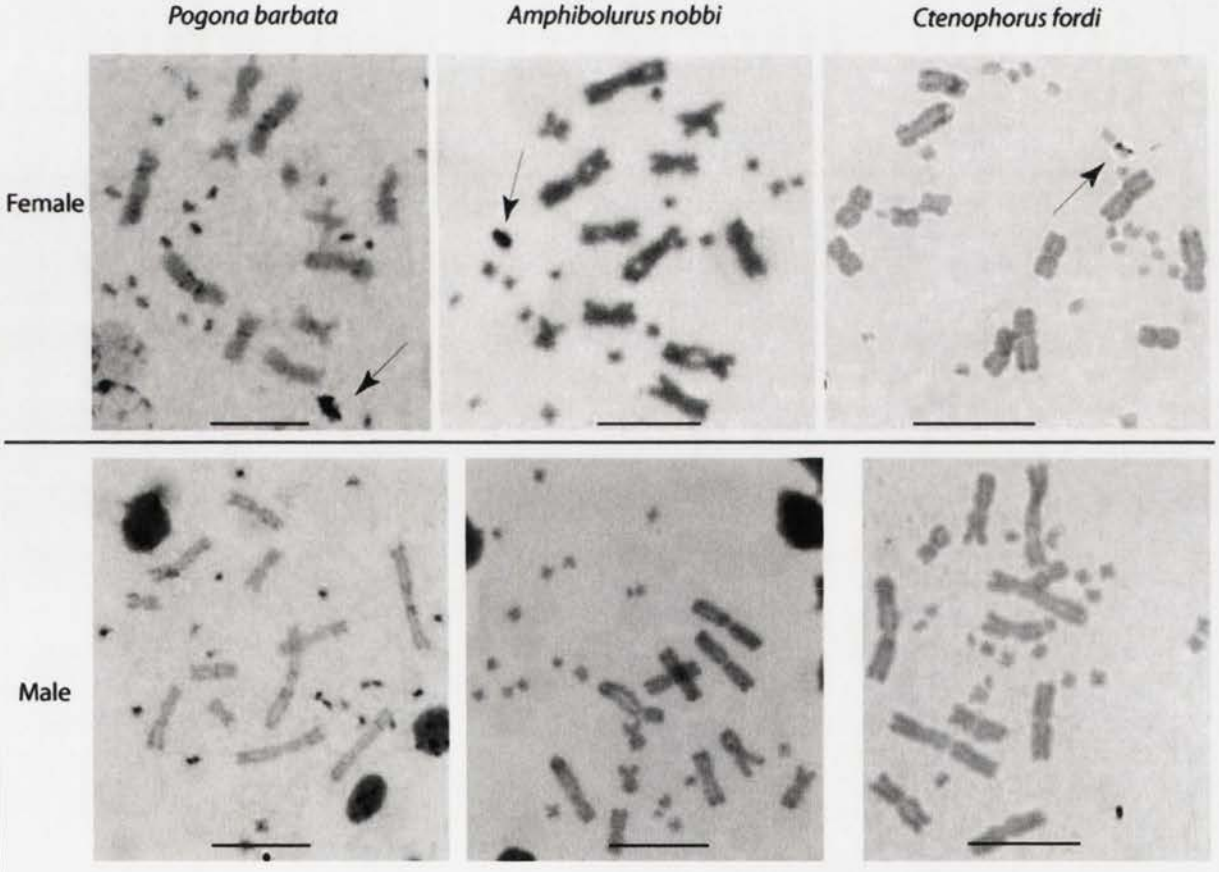

Fig. 1 C-banded metaphase chromosomes in three species of Australian dragon lizards. Arrows indicate heterochromatic W chromosomes in females. Scale bar represents 10 μm

◀ **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.

Acknowledgements We thank T. Uller for providing specimens of *Ctenophorus fordi*. Animal collection, handling, and sampling were performed according to the guidelines of the Australian Capital Territory Animal Welfare Act 1992 (Section 40) and licences issued by the Queensland, South Australia, Victoria, Australian Capital Territory, and New South Wales state governments. All experiments were performed with the approval of the University of Canberra Animal Experimentation Ethics Committee (Proposal CEAE 04/04) and the Australian National University Animal Experimentation Ethics Committee (Proposals R.CG.02.00 and R.CG.08.03). This work is supported by Australian Research Council Discovery Grants (DP0449935 and DP0881196) awarded to J.A.M.G., S. S. and A.G, and S.S. and A.G respectively.

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E I G H T - Synopsis and future work

In the preceding chapters, I used non-model reptiles to address several questions about the evolutionary forces that act on sex chromosomes. These genomic regions are of particular interest to biologists not only because of their role in sex determination, but their special inheritance patterns provide a unique insight to the way evolution shapes genome biology and organisation in all species.

Dosage compensation

I have shown that despite being well conserved and of considerable age, the sex chromosomes of snakes probably do not undergo global dosage compensation. This challenges previous notions that compensatory mechanisms are essential in coping with dosage imbalances brought about by a degraded, gene-poor sex chromosome (Charlesworth 1978; Marín et al. 2000). It also appears that Ohno's Law as regards the conserved gene content of sex chromosomes applies in snakes as well as birds and therian mammals (Ohno 1967); although, perhaps for reasons other than the maintenance of global dosage compensation mechanisms. To bolster this conclusion, further work is required. The extent to which the snake Z is conserved across species could be easily determined by making a snake Z chromosome paint. The same could also be achieved by gene mapping, for example in the Australian Eastern brown snake examined here, however this approach is labour intensive. The garter snake, *Thamnophis sirtalis*, would be an ideal model in which to study snake dosage compensation because it has differentiated sex chromosomes, a BAC library is available, it is favoured for whole genome sequencing and is amenable to captive rearing, so sufficient samples could be obtained easily. Importantly, pedigrees of captive populations would enable the detection of maternal or paternal biases in allele expression and so illuminate sex-specific transcriptional regulation mechanisms.

Sex chromosome degeneration

Ohno's (1967) insightful theory of how sex chromosomes differentiate from autosomal pairs was conceived in large part through observations made of snakes (Beçak et al. 1964). More than 40 years later, snakes have been all but forgotten by those interested in sex determination and sex chromosome evolution. The observation of shared sequences on the sex chromosomes of birds and snakes and greater diversity of repetitive DNA in derived snakes suggests that they may yet provide many more insights into the processes that shape sex chromosome differentiation. In many snakes, it appears that W degeneration occurs rapidly and repeatedly after speciation, in contrast to the pace suggested for other taxa (e.g. Lahn & Page 1999).

Gametolog analysis would be of great interest from a comparative perspective, because evolutionary strata have been suggested for birds and mammals, but have not been examined in snakes where the phylogenetic aspect of differentiation is well characterised. Similarly, different evolutionary rates are known from sex chromosome homologs in birds and mammals, but have not been measured in snakes. Recent mapping of genes to the snake Z and W chromosomes by Matsubara et al. (2006) makes these and many other questions now tractable.

Origins of sex chromosomes

Comparative mapping of genes and sex-linked sequences across reptiles has provided a detailed picture of the sometimes conserved and often dynamic nature of changes in sex determination mechanisms. Variability in sex determining mechanisms is exemplified by lizards, particularly among Agamids. Mapping sex-linked sequences from *Pogona vitticeps* across related species indicates rapid transitions between TSD and GSD and even the evolution of novel sex chromosomes. The sex chromosomes of *P. vitticeps* may even bear some homology to the chicken Z (Ezaz et al. unpub data). The unusual conservation of a sex determining role for the chicken Z in all birds, monotremes, a gecko and a turtle suggests that among amniotes (with the exception of lizards), only snakes and therian mammals have evolved novel sex chromosomes. Mapping genes in more basal snake lineages could push the age of the snake Z back even further than 105 million years. Far from being a living fossil, the tuatara's genome appears to be highly rearranged and attests to the derived state of this species, though more detailed mapping is still required. A fully integrated map of the *Xenopus* genome is nearing completion and will be extremely useful in unravelling the evolutionary history of amniote sex chromosomes.

Sex determining genes

With the exception of therian mammals, birds and two species of fish, primary sex determining genes remain unknown for most vertebrates; none is known from any reptile. The conserved sex chromosome system of snakes makes them the logical choice in which to search for a reptilian equivalent of *SRY*, *DMRT1* or *DMY*. Having established that the snake Z chromosome is equivalent in large part to chicken chromosome 2, the search for targets is narrowed considerably. Sex chromosome anuploidy in snakes, as in birds, is largely unknown so a dominant W or dosage of a Z borne gene could be equally likely. Sporadic parthenogenesis has been reported in a handful of species and depending on the particular meiotic aberration both males and females are produced (Lampert 2008). In the only reported case of facultative triploidy in snakes, an adult cottonmouth viper, *Agkistrodon piscivorous*, had no male

intromittent organs and gonads were underdeveloped, but the karyotype was not determined (Tiersch & Figiel 1991). The only known snake to reproduce by true parthenogenesis, *Ramphotyphlops braminus*, belongs to the basal Scolecophidia (Wynn et al. 1987; Ota et al. 1991). All are triploid females and the presence of a single chromosome with a unique secondary constriction in every animal could be a W sex chromosome (Fig 1). Perhaps, like the Y chromosome of mammals, it bears a dominant gene that directs sexual development down the female pathway.

Fig 1. Karyotype of the brahminy or flowerpot snake, *Ramphotyphlops braminus* (Scolecophidia), adapted from Ota et al (1991). This species has a cosmopolitan distribution and is the only known snake to show true parthenogenesis. A triploid karyotype suggests its all-female population arose by hybridisation. One small chromosome (arrowhead) possesses a secondary constriction that may indicate a dominant W.

A plea for sequence

There is a considerable bias in current genome sequencing projects towards mammals despite the biological diversity and phylogenetic depth within Sauropsida. To date, more than 36 mammals have been or are being sequenced, yet only three non-mammalian amniotes have public genome projects. The chicken and zebra finch are sequenced at depth, with reasonable mapping efforts being undertaken in parallel. The genome of the *Anolis* lizard, however, remains in 'completed draft' form, without a physical or linkage map. Lamentably, physically anchoring sequence scaffolds to chromosomes appears to be an afterthought in many otherwise well conceived genome projects (Lewin et al. 2009). Definitive insights into the evolution of sex chromosomes and genome organisation *per se*, will remain elusive until greater efforts are made to sequence *and map* the genomes of non-mammal amniotes.

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Appendix

Oral Presentations

ANU Graduate Student Conference, Canberra 2009.
Dosage compensation and the evolution of sex chromosomes in Australian snakes

Australasian Evolution Society, Canberra 2009.
Molecular aspects of sex chromosome degeneration in snakes

Applied Ecology Research Group, University of Canberra, Canberra 2009.
Molecular aspects of sex chromosome degeneration in snakes

Department of Ornithology, Museum of Comparative Zoology, Harvard University, Boston 2009.
Sex in Reptiles

Genetic Society of Australasia, Brisbane 2009.
Accumulation of sex chromosome repeats and dosage compensation in Australian snakes

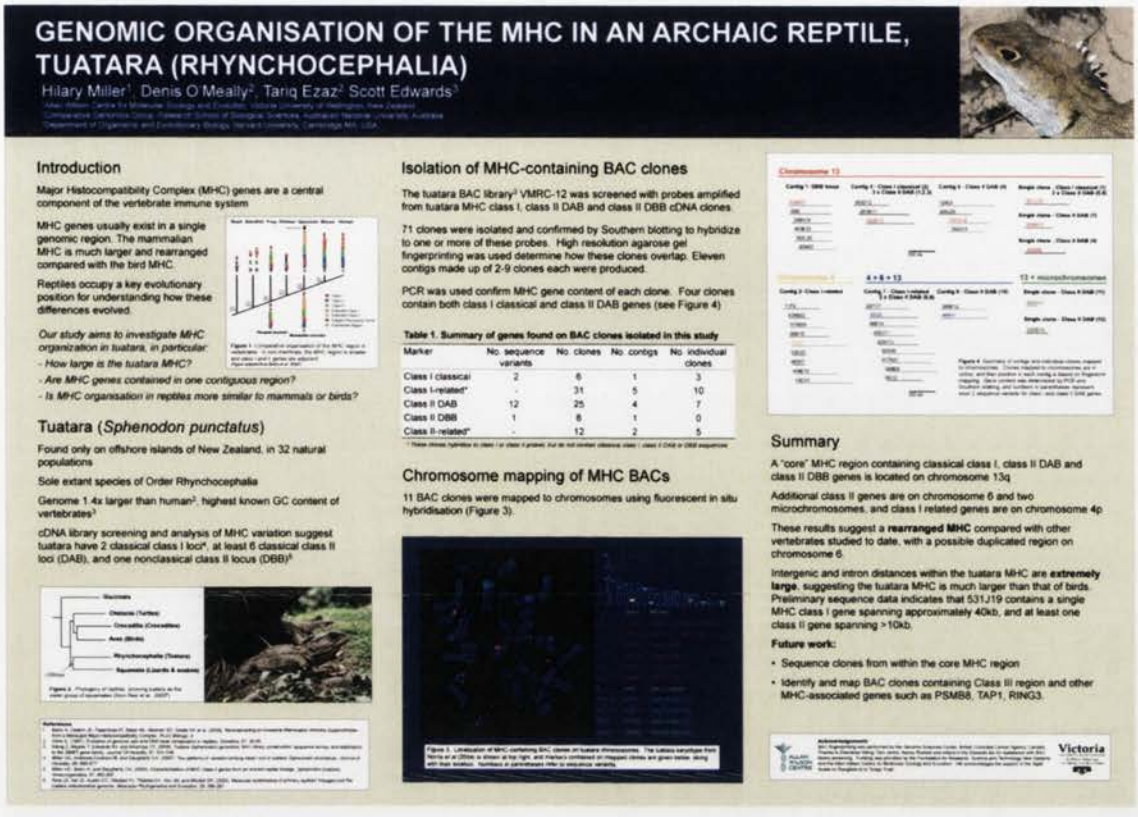
2nd Australian Sex Summit, Flowerdale 2008.
Sex chromosome repeats: insights from snakes and birds.

NARU seminar series, ANU, Darwin 2008.
Sex in reptiles: evolution of reptile sex chromosomes & sex determination mechanisms

Mini-symposium on crocodile genetics and sex determination, University of Sydney, Sydney 2007.
Evolution of reptile sex chromosomes

Poster Presentations

Gordon Research Conference on Ecological & Evolutionary Genomics, New Hampshire, USA 2009.



GENOMIC ORGANISATION OF THE MHC IN AN ARCHAIC REPTILE, TUATARA (RHYNCHOCEPHALIA)

Hilary Miller[1], Denis O'Meally[2], Tariq Ezaz[2] Scott Edwards[3]

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Introduction

Major Histocompatibility Complex (MHC) genes are a central component of the vertebrate immune system

MHC genes usually exist in a single genomic region. The mammalian MHC is much larger and rearranged compared with the bird MHC.

Reptiles occupy a key evolutionary position for understanding how these differences evolved.

Our study aims to investigate MHC organization in tuatara, in particular:

- *How large is the tuatara MHC?*
- *Are MHC genes contained in one contiguous region?*
- *Is MHC organisation in reptiles more similar to mammals or birds?*

Tuatara (*Sphenodon punctatus*)

Found only on offshore islands of New Zealand, in 32 natural populations

Sole extant species of Order Rhynchocephalia

Genome 1.4x larger than human[2], highest known GC content of vertebrates[3]

cDNA library screening and analysis of MHC variation suggest tuatara have 2 classical class I loci[4], at least 6 classical class II loci (DAB), and one nonclassical class II locus (DBB)[5]

Isolation of MHC-containing BAC clones

The tuatara BAC library[3] VMRC-12 was screened with probes amplified from tuatara MHC class I, class II DAB and class II DBB cDNA clones.

71 clones were isolated and confirmed by Southern blotting to hybridize to one or more of these probes. High resolution agarose gel fingerprinting was used determine how these clones overlap. Eleven contigs made up of 2-9 clones each were produced.

PCR was used confirm MHC gene content of each clone. Four clones contain both class I classical and class II DAB genes (see Figure 4)

Table 1. Summary of genes found on BAC clones isolated in this study

Marker	No. sequence variants	No. clones	No. contigs	No. individual clones
Class I classical	2	6	1	3
Class I-related*	-	31	5	10
Class II DAB	12	25	4	7
Class II DBB	1	6	1	0
Class II-related*	-	12	2	5

* These clones hybridize to class I or class II probes, but do not contain reference class I class II DAB or DBB sequences

Chromosome mapping of MHC BACs

11 BAC clones were mapped to chromosomes using fluorescent in situ hybridisation (Figure 3).

Summary

A "core" MHC region containing classical class I, class II DAB and class II DBB genes is located on chromosome 13q

Additional class II genes are on chromosome 6 and two microchromosomes, and class I related genes are on chromosome 4p

These results suggest a **rearranged MHC** compared with other vertebrates studied to date, with a possible duplicated region on chromosome 6.

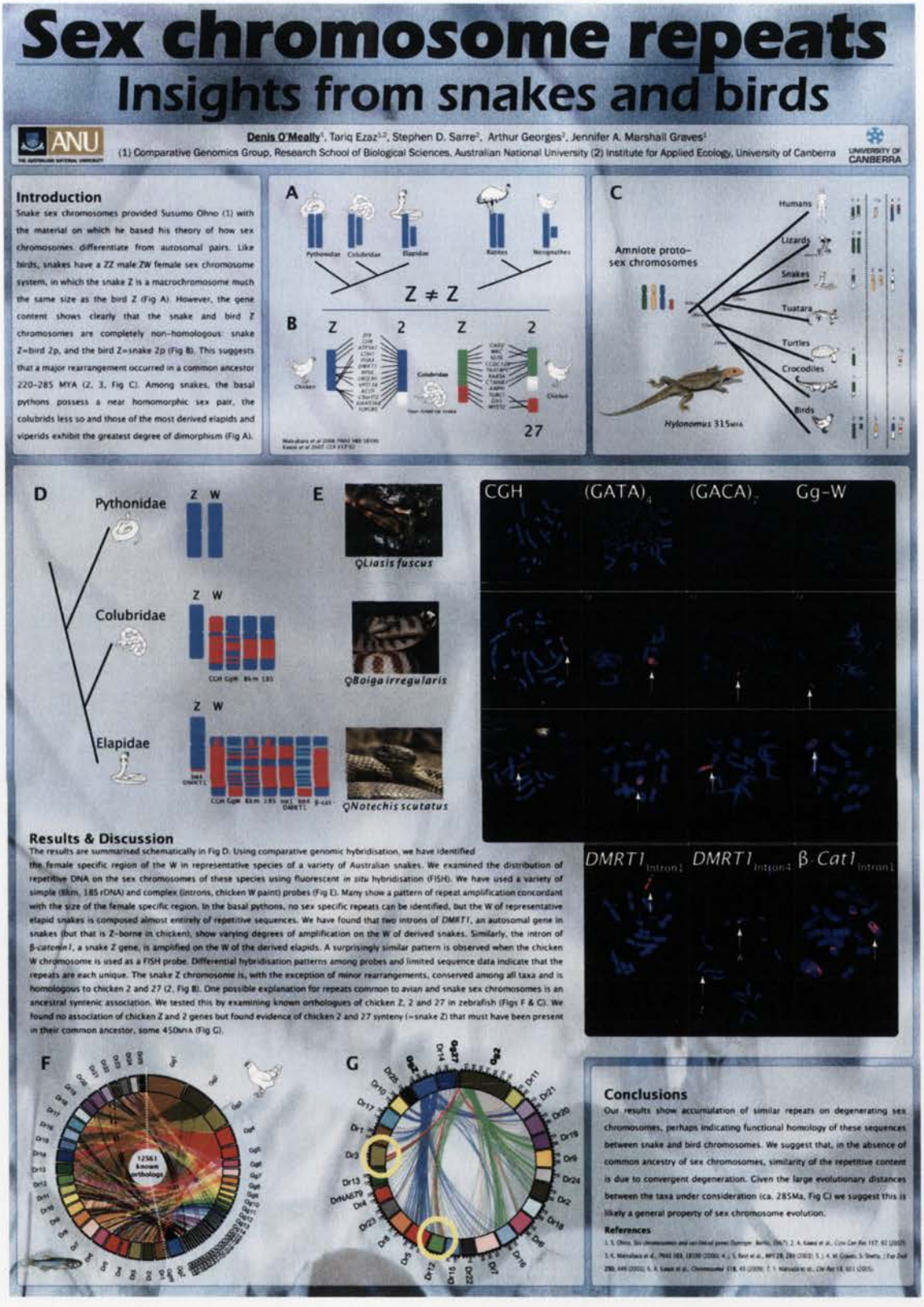
Intergenic and intron distances within the tuatara MHC are **extremely large**, suggesting the tuatara MHC is much larger than that of birds. Preliminary sequence data indicates that 531J19 contains a single MHC class I gene spanning approximately 40kb, and at least one class II gene spanning >10kb.

Future work:

- Sequence clones from within the core MHC region
- Identify and map BAC clones containing Class III region and other MHC-associated genes such as PSMB8, TAP1, RING3.

Acknowledgements

5th International Symposium on Vertebrate Sex Determination. Hawaii 2009.



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

Tariq Ezaz[1,2], Alexander E. Quinn[2], Stephen D. Sarre[2], **Denis O'Meally**[1], Arthur Georges[2], Jennifer A. Marshall Graves[1]
1 Comparative Genomics Group, Research School of Biological Sciences, Australian National University
2 Institute for Applied Ecology, University of Canberra

Introduction

Sex in vertebrates is determined either by genes on sex chromosomes (Genotypic Sex Determination, GSD) or by the influence of temperature on the developing embryo (Temperature-dependent Sex Determination, TSD). Species with GSD have either male heterogamety (XY males, XX females) or female heterogamety (ZW females, ZZ males). In dragon lizards (Agamidae) both GSD and TSD systems are found, and in some species both systems interact to determine sex. The close evolutionary relationship of Australian dragon lizards (ca. 25MYA, Hugall *et al* 2008) and their rapid radiation makes them an ideal group in which to study transitions between GSD and TSD. Karyotypes are highly conserved within the group but sex chromosomes are indistinguishable by standard cytological staining techniques in GSD species. We have previously identified the sex chromosomes of one species, *Pogona vitticeps*, by CGH and C-banding (Ezaz *et al* 2005) and isolated sex-linked sequences by AFLP analysis (Quinn *et al* 2007).

Methods

A novel 3kb DNA fragment ("PvZW3") from the sex chromosomes of the central bearded dragon, *Pogona vitticeps*, was isolated by AFLP (Quinn *et al* 2007) and subsequent genome walking procedures. Cloned PCR products were labelled by nick translation incorporating fluorescently tagged dUTP. The probe was tested in *P. vitticeps* by fluorescent *in situ* hybridisation (FISH) onto metaphase chromosomes (Fig 1). We used the probe to screen both GSD and TSD dragons by FISH (Fig 2). One female from four species was subsequently C-banded to confirm hybridisation to sex chromosomes (Fig 2).

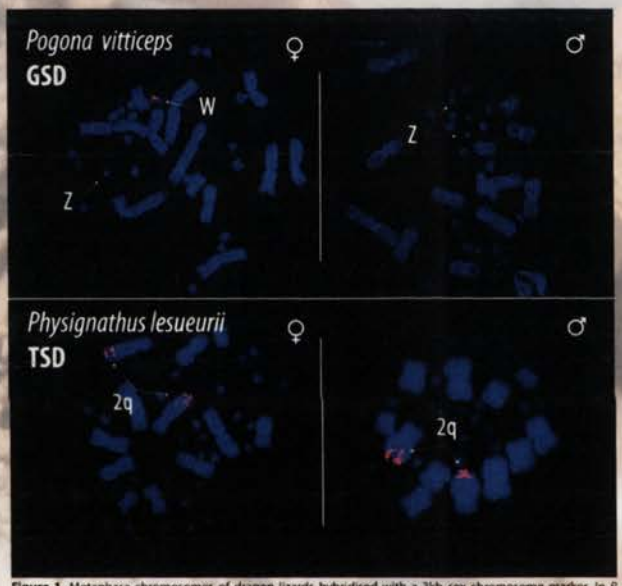


Figure 1. Metaphase chromosomes of dragon lizards hybridised with a 3kb sex chromosome marker. In *P. vitticeps*, differential hybridisation to the Z and W micro-chromosomes of the female is evident. In *P. lesueurii* the hybridisation signal localises to the distal end of 2q and no sex-specific differences are obvious. In all other species examined hybridisation was only observed on a pair of microchromosomes, as for *P. vitticeps* (see Fig 2).

Results & Discussion

Hybridisation of a 3kb sex chromosome probe on a single pair of chromosomes in each species examined indicates that sex chromosome sequences are conserved, implying homologous chromosomes between GSD and TSD dragons. Our findings revealed that with the exception of two species, the same microchromosome pair is retained in GSD and TSD dragons, suggesting it is sex specific in GSD species (Fig 2). In *C. fordii*, PvZW3 hybridisation is discordant with W chromosome C-banding, suggesting that in this species a novel sex microchromosome pair has evolved. The macrochromosomal location of PvZW3 in the more basal *P. lesueurii* suggests a chromosomal rearrangement (Fig1). We will establish the ancestral arrangement by investigating outgroup species, particularly basal Asian fauna. Sex chromosome homology will be corroborated by FISH with specific sex chromosome paints. Our findings suggest that both the mode of sex determination and the sex chromosomes are in state of flux, providing evidence of multiple evolutionary transitions. With the increasing availability of genomic resources Australian dragon lizards are well placed to provide unique insights into the evolution of sex determination mechanisms.

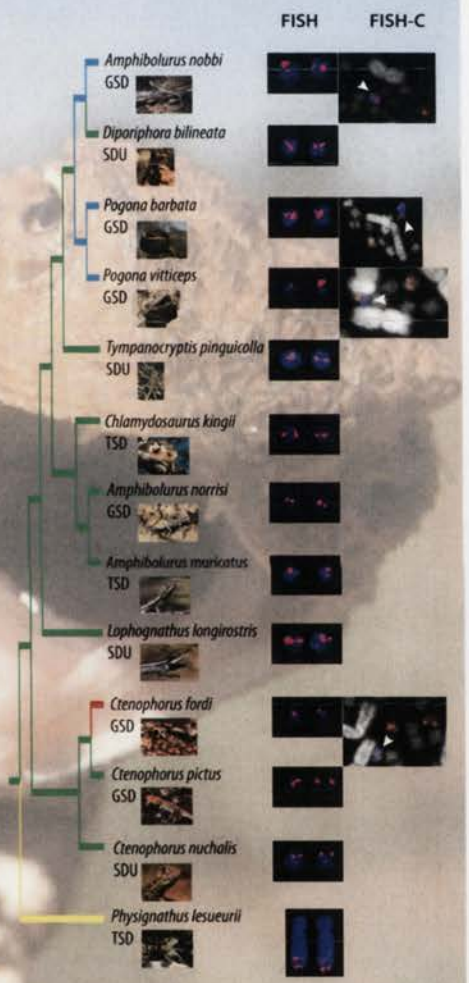


Figure 2. Phylogeny of dragon lizard species included in this study showing physical mapping of PvZW3 in thirteen species and subsequent FISH and C-banding in four species (pruned tree modified after Hugall *et al* 2008). Where PvZW3 hybridisation is concordant with C-banded microchromosomes, the branch is indicated by blue; red indicates a discordant microchromosomal location; and [illegible] indicates PvZW3 hybridised to a macrochromosome. Arrows indicate pseudocoloured C-banded W chromosomes; red hybridisation signals represent the location of PvZW3. Sex determination (TSD or GSD) after Harlow (2004) and unpublished data (T. Ezaz *et al*); SDU - sex determination unknown. Images were captured, analysed, pseudo-coloured and merged (including C-bands in merged C-FISH images) using IPlab (Scanalytics, USA).

References: **Ezaz** *et al* 2005 *Chromosome Research* **13**: 763. **Harlow** 2004 In: *Temperature dependent sex determination in vertebrates*, Valenzuela & Lance Eds. **Hugall** *et al* 2008 *Biological Journal of the Linnean Society* **93**: 343. **Quinn** *et al* 2007 *Science* **316**: 411
Acknowledgements: This project is funded by ARC Discovery grants (DP0449935, DP0881196). Animals were collected under scientific licences from NSW (S12364), ACT (LO2006220), NT (26791), SA (E25029) & Vic (10003441) with the approval of local ethics committees (ANU E.CG.02.00 & R.CG.08.03; UC CEA04/04). Photos: Museum Victoria, Cogger 2000 *Reptiles and amphibians of Australia*; ABC Online, ACT Government, D. O'Meally & J. Wilson ANU.

Genetics Society of Australasia, University of Adelaide 2008. (Student Prize Winner)

ZooFISH reveals homologous sex chromosomes among GSD and TSD Australian dragons

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Introduction

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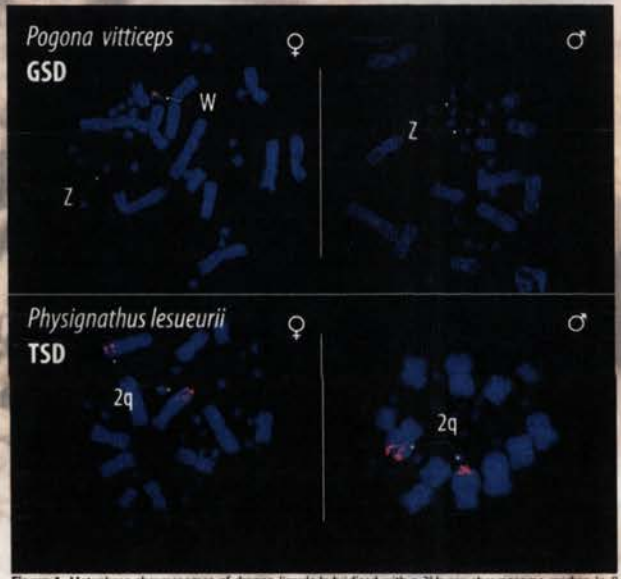

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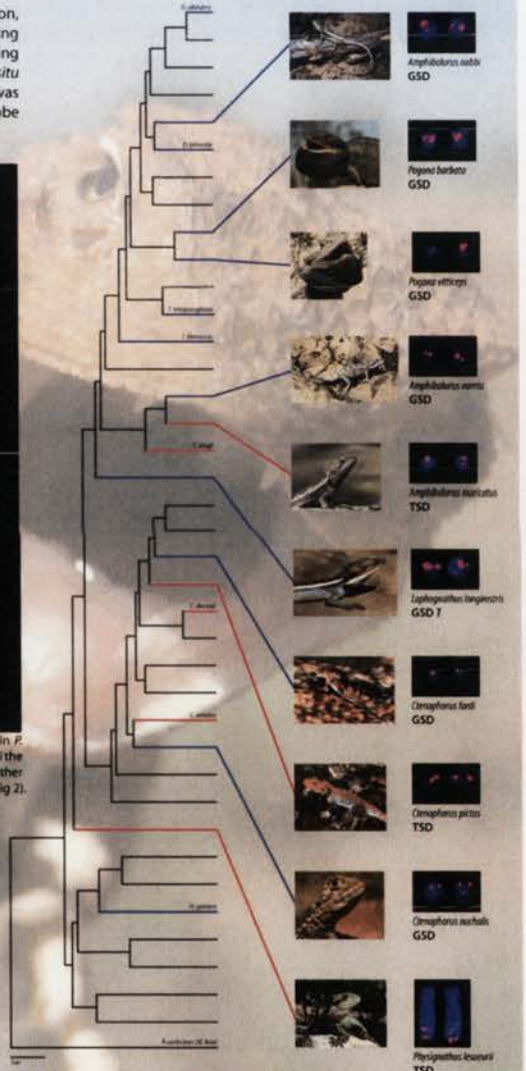

Figure 2. Molecular cladogram of Australian agamids and allies, showing the hybridisation pattern of a 3kb sex chromosome probe on species examined in this study (modified after Hugall *et al* 2008). Sex determination (TSD or GSD) after Harlow (2004) and unpublished data (T. Ezaz *et al*)

References: Ezaz *et al* 2005 *Chromosome Research* **13**: 763, Harlow 2004 In: *Temperature dependent sex determination in vertebrates*, Valenzuela & Lance Eds. Hugall *et al* 2008 *Biological Journal of the Linnean Society* **93**: 343, Quinn *et al* 2007 *Science* **316**: 411
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Tuatara *DMRT1*: duplication or separation?

ANU
Denis O'Meally[1], Tariq Ezaz[1], Steve Sarre[2], Arthur Georges[2] and Jenny Graves[1]
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[2] Institute for Applied Ecology, University of Canberra, Bruce, ACT

Introduction

Tuatara - an ancient lineage

Tuatara, *Sphenodon* sp., are the last remaining representatives of a once widespread reptilian lineage. The only two extant species (*S. punctatus* and *S. guntheri*) are now confined to several off-shore islands of New Zealand. They possess a number of peculiarities of biology, including the lowest active body temperature of any reptile, the presence of a vestigial parietal eye, a bird-like egg-tooth and a 13 month incubation period, among many others. Modern tuatara have changed little in body form from their 225MY old ancestors, and are fittingly known as 'living fossils'.

Fig 1. Though once widespread, tuatara are now restricted to several off-shore New Zealand islands. Two closely related species have been described, *S. punctatus* shown in red and *S. guntheri* in yellow. Open circles indicate translocated populations. **Fig 2.** *S. punctatus* from Stephen's Island.

Mapping tuatara DMRT1

BACs containing *DMRT1* map to two pairs of microchromosomes in tuatara. Chromosome painting reveals that three different pairs of macrochromosomes share homology with the Chicken Z.

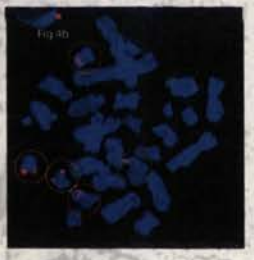

Fig 4a. We mapped previously published[1], commercially supplied BAC clones (SymBio Corp., California) containing one or more genes of the tuatara *DMRT* family to tuatara metaphase chromosomes. **Fig 4b.** In a separate experiment, commercially available chicken Z chromosome paint (Farmachrom, Canterbury, UK) was hybridised to tuatara metaphase chromosomes under low stringency conditions. Pairs of chromosomes are indicated by coloured circles. The chicken Z paint may represent repetitive elements as no attempt was made to suppress hybridisation of such sequences.

DMRT1 is a uniquely conserved gene

DMRT1 (Doublesex- and mab3-related transcription factor 1) is conserved throughout metazoans and is implicated in sex determination and differentiation in a multitude of species, from *Drosophila* to mice. In humans it is autosomal and deletion of one copy results in XY sex reversal. In birds, it is the best candidate for a Z borne primary sex determinant. It is a member of the DM domain gene family and in all animals examined to date, the order of the first three genes in the family is preserved (Fig 3).

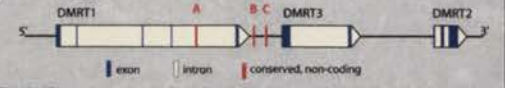

Fig 3. *DMRT1* contains a novel zinc finger domain (DM domain) and exhibits sex-specific expression in vertebrates and invertebrates. It is always found in a contiguous block with two other members of the gene family, DMRT2 and DMRT3. Interspersed are conserved, non-coding regions with putative regulatory roles.

Sex chromosome homology

Recent gene mapping studies have shown that sex chromosomes evolved many times among vertebrates. Homology can be inferred even in animals without sex chromosomes, such as tuatara, by investigating gene content. We are using BACs, degenerate primers, cloned mRNAs and chromosome paints to investigate chromosome homology in representative reptiles and birds.

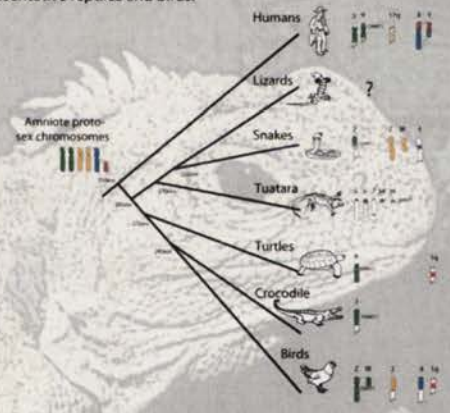

Fig 5. Several recent mapping[2,3,4] and painting[5] studies have demonstrated the relationships among reptilian sex chromosomes or their counterparts in species where sex is determined by temperature. Notably, the arrangement in tuatara remains unknown. In addition to chromosome paints, we are mapping *DMRT1, ATP5A1, GNR, CDH1, DMRT1, RPS6, ACOT* in representative reptiles using FISH. Divergence dates after Rest et al[6].

Conclusion

Was the ancestral tuatara tetraploid? Have members of the *DMRT* family been separated in tuatara or has the entire block been duplicated onto another chromosome?

Either situation would be unique to tuatara among all amniotes studied to date. Determining the genomic context of *DMRT1* will answer the question of whether duplication of this important gene has occurred in tuatara or if it has been separated from its otherwise ubiquitous partners.

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2. Matsuda et al (2005) *Chrom Res* **13**:601-605
3. Matsubara et al (2006) *PNAS* **103**:18190-18195
4. Kawai et al (2007) *Cytogenet Genome Res* **117**:92-102
5. Graves & Shetty (2001) *J Exp Zool* **290**:449-462
6. Rest et al (2003) *Mol Phyl Evol* **29**:289-297

Acknowledgements
We are grateful to Taronga Zoo, Sydney for access to their tuatara. Blood samples were collected under AEC protocol 3b/04/07. Tuatara images are from http://www.ryanphotographic.com.

Are sequences conserved between the sex chromosomes of birds and snakes?

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2 Institute for Applied Ecology, University of Canberra, Bruce, ACT

Introduction

Sex determination among vertebrates is highly variable and different mechanisms have evolved independently many times (Fig 1). H.J. Müller (variously) first proposed that sex chromosomes evolve from a recombining pair of autosomes, and the heteromorphic member degrades due to the gain of a sex-specific allele, loss of gene function, accumulation of heterochromatin and finally suppression of recombination. S. Ohno supposed he could observe such degeneration (in gross morphology) over evolutionary time amongst the families of snakes. In this group of reptiles, the ancient boids possess near homomorphic gonosomes, the colubrids less so, and those of the most derived elapids and viperids exhibit the greatest degree of dimorphism. In birds, where female heterogamety is similarly ubiquitous, a pattern of W degeneration over evolutionary time is also observed (Fig 2). A recent gene mapping study (Matsubara et al 2006) however, established that the avian Z chromosome shares large regions of homology with Chromosome 2 of snakes, contradicting the hypothesis of common ancestry of snake and bird sex chromosomes (Beçak et al 1964). We used comparative chromosome painting to investigate the relationship of snake and bird sex chromosomes.

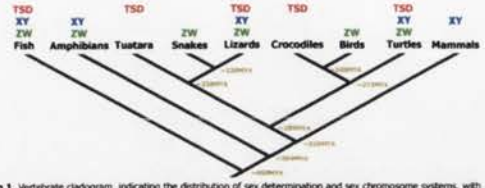

Fig 1. Vertebrate cladogram, indicating the distribution of sex determination and sex chromosome systems, with approximate divergence times. Birds and snakes shared a common ancestor some 285MYA. TSD = temperature-dependant sex determination; XY, ZW = Genetic-dependent sex determination, with male and female heterogamety respectively.

Aims

To:

- Investigate homology of bird and snake sex chromosomes using cross-species chromosome painting (ZooFISH)
- Characterise the genic and repetitive content of the snake W chromosome
- Investigate similarities in the degeneration of bird and snake W chromosomes

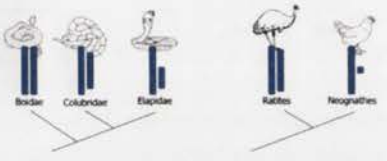

Fig 2. Degeneration of W chromosomes in snakes and birds. In ancient lineages, sex chromosomes are near homomorphic. In derived lineages the W chromosome tends towards degeneration and is morphologically distinguishable from the Z chromosome.

ZooFISH

- *Individual chromosomes were isolated by flow cytometry or microdissection*
- *We labelled chromosome probes with a fluorochrome by nick translation, DOP-PCR or other whole genome amplification techniques*
- *Probes were hybridised to denatured metaphase chromosomes, revealing regions of homology*
- *We screened a variety of bird and snake spreads with a chicken Z and W specific paints*
  - **Chicken Z does not hybridise to snake chromosomes**
  - **We found no hybridisation of chicken W among ancient lineages, but it hybridises to both the derived bird and snakes**

Fig 3. Metaphase chromosomes of a ratite (*Dromaius novaehollandiae*, Dn), two neognathes (*Gallus gallus*, Gg; *Platycercus elegans*, Pe), a boid (*Liasis fuscus*, Lf), a colubrid (*Elaphe quadrivittata*, Eq) and an elapid snake (*Notechis scutatus*, Ns) hybridised with chicken W chromosome paint. We found hybridisation on a single small microchromosome of the crimson rosella (Pe), presumably indicating the highly degraded W. Interestingly no hybridisation was found on either emu (Dn) or water python (Lf), whose sex chromosomes are homomorphic. The GgW paint hybridises to a large region of the colubrid (Eq) W and to a lesser degree with the degenerate, acrocentric W of the elapid (Ns). No hybridisation was found when the GgZ paint was used on snakes.

Painting

Bkm repeat

- *Banded krait minor satellite is a sex specific repeat first isolated from the banded krait,* Bungarus fasciatus
- *Has been found in the heterogametic sex of many vertebrate species, including humans (by Southern blot analysis)*
- *We screened representatives of divergent bird and snake families with $(GATA)_4$- and $(GACA)_7$-Cy3 labelled probe*
  - **We found no hybridisation among birds**
  - **Among snakes, *Bkm* accumulation is correlated with W chromosome degeneration**

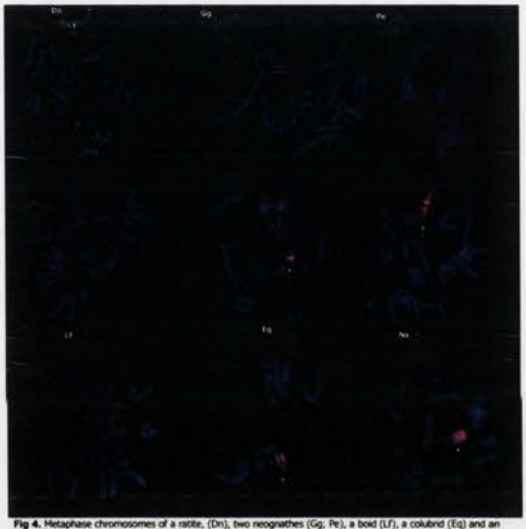

Fig 4. Metaphase chromosomes of a ratite, (Dn), two neognathes (Gg; Pe), a boid (Lf), a colubrid (Eq) and an elapid (Ns) hybridised with *Bkm* probes. There is a total absence of hybridisation among the birds and ancient boids, and a trend towards increasing accumulation in the degenerated W of the colubrid and elapid snakes. The distribution of *Bkm* on the W chromosome of the derived snakes differs from that of the GgW paint, suggesting that they constitute two distinct classes of repeats.

Common ancestry or convergent degeneration?

- Our results show accumulation of similar repeats on degenerating chromosomes, suggesting functional homology between snake and bird sex chromosome degradation
- Chicken W chromosome appears to have a novel class of repeats, distinct from *Bkm*
- Lack of chicken Z hybridisation on snake chromosomes does not favour the common ancestry of avian and reptilian sex chromosomes

What next?

- Characterise the sequence of chicken W repeats by either
  - Southern blot analysis of snake genomic DNA, using the chicken W paint as probe
  - Screening a chicken BAC library with snake W paints and subsequent physical mapping
  - Comparative sequence analysis of chicken Z and W chromosomes using the chicken genome assembly.

Acknowledgements

We are grateful to K. Matsubara for providing Japanese rat snake chromosomal material and H. Tempest for production of the *Gallus gallus* chromosome paints. Several past members of the CGG laboratory have contributed enormously in the production of cell lines. DOM is supported by an ANU PhD Scholarship and in part by an ARC Discovery Grant.

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