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

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Abstract Snake sex chromosomes provided Susumo Ohno 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. We used comparative genomic hybridization to identify the female-specific region of the W chromosome in representative species of Australian snakes. Using this approach, we show that an increasingly complex suite of repeats accompanies the evolution of W chromosome heteromorphy. In particular, we found that while the python *Liasis fuscus* exhibits no sex-specific repeats and indeed, no cytologically recognizable sex-specific region, the colubrid *Stegonotus cucullatus* shows a large domain on the short arm of the W chromosome that consists of female-specific repeats, and 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 *N. 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 ancestral but as yet undetected shared synteny of bird and snake sex chromosomes or may indicate functional homology of the repeats and suggests that degeneration is a convergent property of sex chromosome evolution. 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.

Key words Aves · Bkm · degeneration · evolution · Serpentes · sex chromosomes · repeats

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Abbreviations

BAC	Bacterial artificial chromosome
Bkm	Banded krait minor satellite DNA
CGH	Comparative genomic hybridization

DAPI	4′,6-diamidino-2-phenylindole
DOP-PCR	Degenerate oligonucleotide primed polymerase chain reaction
dUTP	2′-deoxyuridine 5′-triphosphate
FISH	Fluorescence in-situ hybridization
GGA	*Gallus gallus*
HSA	*Homo sapiens*
MSY	Male-specific region on the Y chromosome
MYA	Million years ago
NOR	Nucleolus organizer region
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
SSC	Standard saline citrate
v/v	Volume/volume
w/v	Weight/volume
XTR	*Xenopus tropicalis*

Introduction

Sex chromosomes differentiate after 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 the accumulation 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 the restriction of those loci to one sex by suppression of recombination, which may subsequently spread along most or all of the chromosome (Nei 1969; Charlesworth and 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 and Steinemann 2005).

Most sex chromosome repeats are species-specific and highly variable, even between closely related taxa (Tone et al. 1984; Itoh and Mizuno 2002; Yoshido et al. 2007; Vítková et al. 2007; Hughes et al. 2010). This suggests that rapid amplification and molecular differentiation occur repeatedly after divergence from a common ancestor. Sequencing analysis 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 only 6 MYA (Skaletsky et al. 2003; Hughes et al. 2010). The MSY regions of both species have undergone significant rearrangement, such that the structure in a common ancestor cannot be inferred. The chimp MSY has several unique palindromic structures that are larger in size and has multiple copies 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, such as the *P2000-17*, *Apa*I, and *ZMB* repeat families isolated from bird W chromosomes (Griffiths and Holland 1990; Yamada et al. 2006; Itoh et al. 2008), have a broader taxonomic distribution, but their lineage-specific amplification suggests that stochastic processes play an important role. Nucleolus organizer regions (NORs) consist of tandem arrays of 18S, 5.8S, and 28S rDNA (Shaw and 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). Another repeat, the banded krait minor-satellite (*Bkm*), first isolated from snakes, 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, including the human Y chromosome (Jones and Singh 1981; Singh et al. 1981; Arnemann et al. 1986; Schäfer et al. 1986; Nanda et al. 1990, 1991; Parasnis et al. 1999). Intriguingly, the frequency of *Bkm* repeats in the snake genome corresponds with the degree of ZW differentiation (Jones and 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 inferred that the progressive stages in sex chromosome degeneration could be observed among the representative families of snakes. 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 content of heterochromatin. Elapidae and Viperidae generally possess markedly dimorphic sex chromosomes, which may differ greatly in size, centromere position, and heterochro-

matin. A similar pattern of progressive degeneration has been described in many other taxa, including mammals (Graves 2006), fish (Kirpichnikov 1981; cited in Rice 1996), and birds (Graves and Shetty 2001; Tsuda et al. 2007; Mank and 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 (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 gene mapping data show that this is not so (Matsubara et al. 2006; Kawai et al. 2007). The chicken Z is homologous to parts of chromosome 2 in snakes, whereas the snake Z is homologous to the short arm of chicken chromosome 2 and chromosome 27. Synteny of 11 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 suite 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 ancestral but as yet undetected shared synteny of bird and snake sex chromosomes. Alternately, such unusual similarity may indicate functional homology of the repeats and suggests that degeneration is a convergent property of sex chromosome evolution.

Materials and methods

Animals

We included in this study representative and common species of Australian snakes. We chose the water python *Liasis fuscus* (Pythonidae); the slaty gray snake, *Stegonotus cucullatus* (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 and Bull 1977; Mengden and Stock 1980; Mengden 1982). Numbers and collection localities are given in Table 1. We used female chicken metaphase spreads 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 to 4 days in 5% CO_2. Fibroblast cultures were established according to the protocol of Ezaz et al. (2009a, b). Chromosomes were harvested 2 h after adding colcemid (75 ng/mL) by treating with hypotonic solution (KCl, 0.075 M) 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 and Russell 2001). DNA concentration was measured on a NanoDrop (Thermo Scientific) and labeled by nick translation with Orange-dUTP (Abbott Molecular) for female DNA and with Green-dUTP for

Table 1 Material examined

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

Collection localities are within Australia

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 200 μM of 6 MW primer (5′-CCG ACT CGA GNN NNN NAT GTG G-3′) (Telenius et al. 1992), 10 μL of ×5 buffer with MgCl2, 20 mM dNTPs, and 1 U of GoTaq polymerase (Promega) in 50 μL. 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.

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

We used a BAC clone (AGI 329J14) from the tammar wallaby (*Macropus eugenii*) containing 18S rDNA (Haines 2005) to map 18S rDNA in *N. scutatus*. The clone was grown in a 15-mL 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).

We made PCR probes in an attempt to map the genomic location of *DMRT1* and *CTNNB1* in *N. scutatus*. Homologous probes for *DMRT1* (intron 1) and *CTNNB1* (terminal intron) were prepared by long range PCR for hybridization to *N. scutatus* chromosomes. Genomic DNA 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 and DMIR for *DMRT1* intron 1 and CTNNB1F and 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. Amplicons were isolated and subject to a second round of PCR to ensure that 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) *Escherichia coli* (Stratagene) according to standard protocols (Sambrook and 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 hybridization, 200–500 ng of labeled DNA and 1 μg boiled gDNA (not used for CGH and *Bkm* hybridization) were co-precipitated and resuspended in hybridization buffer (50% *v*/*v* deionized formamide, 10% *w*/*v* dextran sulfate, ×2 SSC, ×1 Denhardt's solution, and 40 mmol/L sodium phosphate). Hybridization, slide preparation, image capture, and analysis followed the protocol of O'Meally et al. (2009).

Table 2 Oligonucleotide primers used for PCR and sequencing of *DMRT1* intron 1 and the terminal intron of *CTNNB1*

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

See text for product sizes and reaction conditions. Sequencing primers are indicated by a numerical suffix

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 arrangement of genes found on the sex chromosomes of snakes is unique to Serpentes, we examined the arrangement of these regions in chicken, human, and frog. The same approach was used to test for synteny of genes on the Z chromosomes of snakes and chicken in a tetrapod ancestor. We made use of the *Xenopus tropicalis* (XTR) integrated linkage map (Hellsten et al. 2010; A. Sater, personal communication) and *Xenopus*-human and *Xenopus*-chicken annotated orthologs from Ensembl (Vilella et al. 2009). We were able to assign scaffolds bearing *Xenopus*-human orthologs (7841 genes) and *Xenopus*-chicken (6158 genes) to *Xenopus* chromosomes. We inferred ancestral synteny of chicken (GGA) chromosomes by counting *Xenopus*-chicken orthologs on each XTR chromosome for GGA2 and GGAZ to construct a table of the distribution of genes in each species. A Chi-square contingency analysis was used to demonstrate significant departure from a random and independent distribution of GGA orthologs across XTR chromosomes (after Smith and Voss 2007). Departure from the Chi-square expectation in this context can arise from either lack of randomness (ancestral signal) or lack of independence (random translocation of syntenic blocks) or both.

A significant association between the chromosomal location of GGA genes and their orthologs on XTR chromosomes was followed by a test of the significance of individual cells in the table. Standardized residual deviations from expectation were calculated for each cell and tested for significance using a *z* test, following the procedures outlined in Hays (1994:860). The level of significance was adjusted using a Bonferoni correction to contain the overall error rate at or below 0.05. This procedure was repeated for comparisons between human (HSA) and XTR.

Results

Comparative genomic hybridization

CGH revealed the sex-specific region of the W chromosome in females of representative colubrid and elapid snakes but not in the python (*L. fuscus* Fig. 1a–c) for which sex chromosomes are yet to be identified. The sex chromosomes of *S. cucullatus* (Colubridae), the fourth largest pair (Mengden 1982), are submetacentric. CGH clearly identified the W chromosome in that species as heavily biased with female-specific sequences along its whole length but consistently concentrated on the distal long arm (Fig. 1b). The sex chromosomes in *N. scutatus* are the fourth largest pair, and the W is large and acrocentric (Shine and Bull 1977; Mengden 1981). In that species, we found female-specific sequences along most of the length of the W chromosome with the exception of the DAPI-bright distal long arm, which is not marked by the CGH probe (Fig. 1c).

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 hybridized the chicken W chromosome paint to chromosomes of representative snake species (Fig. 1d–f). 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 showed 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 contrast, the distal region of the long arm of the *S. cucullatus* W chromosome showed noticeable accumulation of both classes of sequences, but with

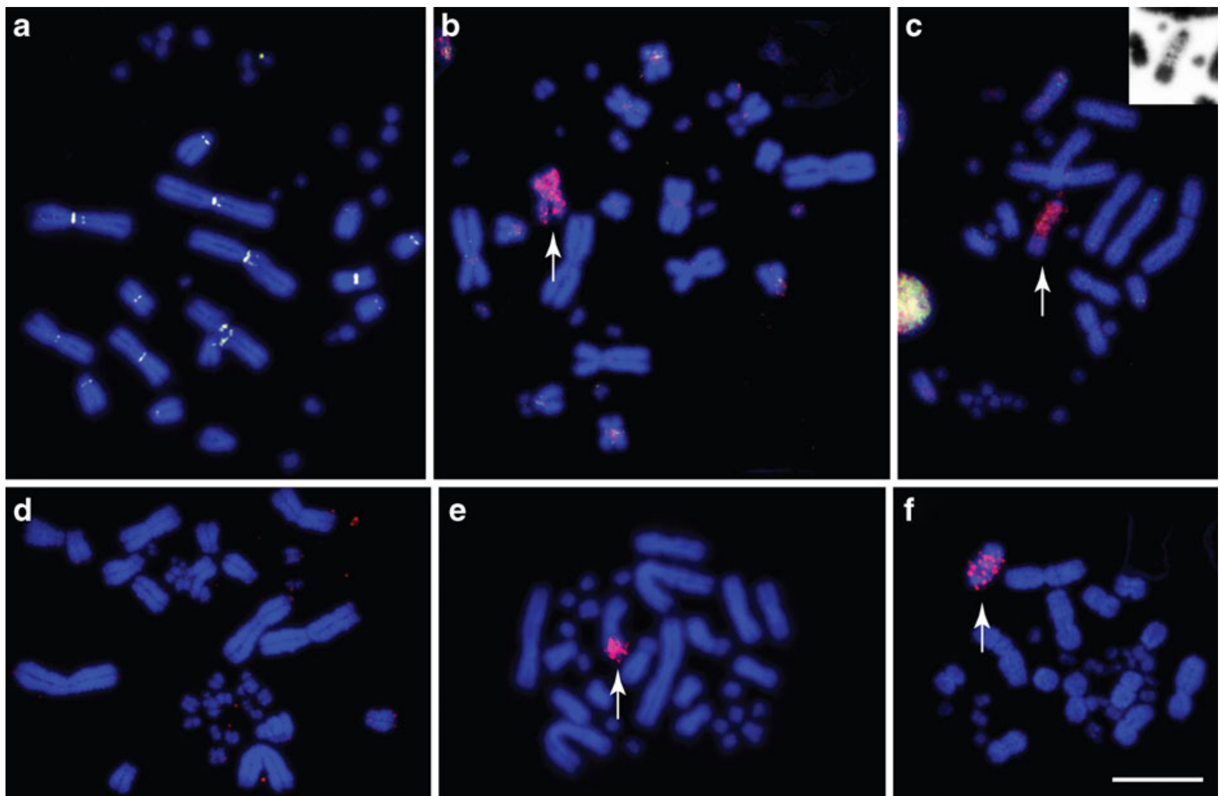

Fig. 1 Comparative genomic hybridization (*upper images*) of male (*green*) and female (*red*) genomic DNA and hybridization of chicken W chromosome paint (*lower images*) to female metaphase spreads of **a**, **d** *L. fuscus*, **b**, **e** *S. cucullatus*, and **c**, **f** *N. scutatus*. The *inset* in **c** shows the inverted DAPI stained W chromosome image of *N. scutatus*. In **a**–**c**, the signals from both channels have been attenuated so that the amplified repeats are emphasized, highlighting the differences between male and female genomes. *Arrows* indicate the W chromosome, and the *scale bar* represents 10 μm

different hybridization pattern and signal intensity. In *N. scutatus*, the W chromosome showed 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 *N. scutatus*

We hybridized an 18S rDNA probe from the tammar wallaby to the metaphase chromosomes of male and female *N. scutatus* (Fig. 3a, b). A single active NOR was previously identified by silver staining at a secondary constriction on chromosome 1 in this species (Mengden 1981). 18S rDNA maps to the same secondary constriction on chromosome 1 in both males and females. In females, however, the W chromosome also showed massive amplification of 18S rDNA sequences along the length of the female-specific region. Hybridization of the two PCR-derived intron probes showed similar amplification on the W.

The *DMRT1* intron 1 probe did not mark chromosome 2, to which this gene is expected to map. Instead, it produced an intense signal along the female-specific region of the W (Fig. 3d). The phage-derived *DMRT*-related probe marked subtelomeric regions on Zp and Wq (Fig. 3e). Hybridization on the W chromosome corresponded 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 as described by Brunner et al. (2001) (data not shown); and Stiglec (2007) amplified

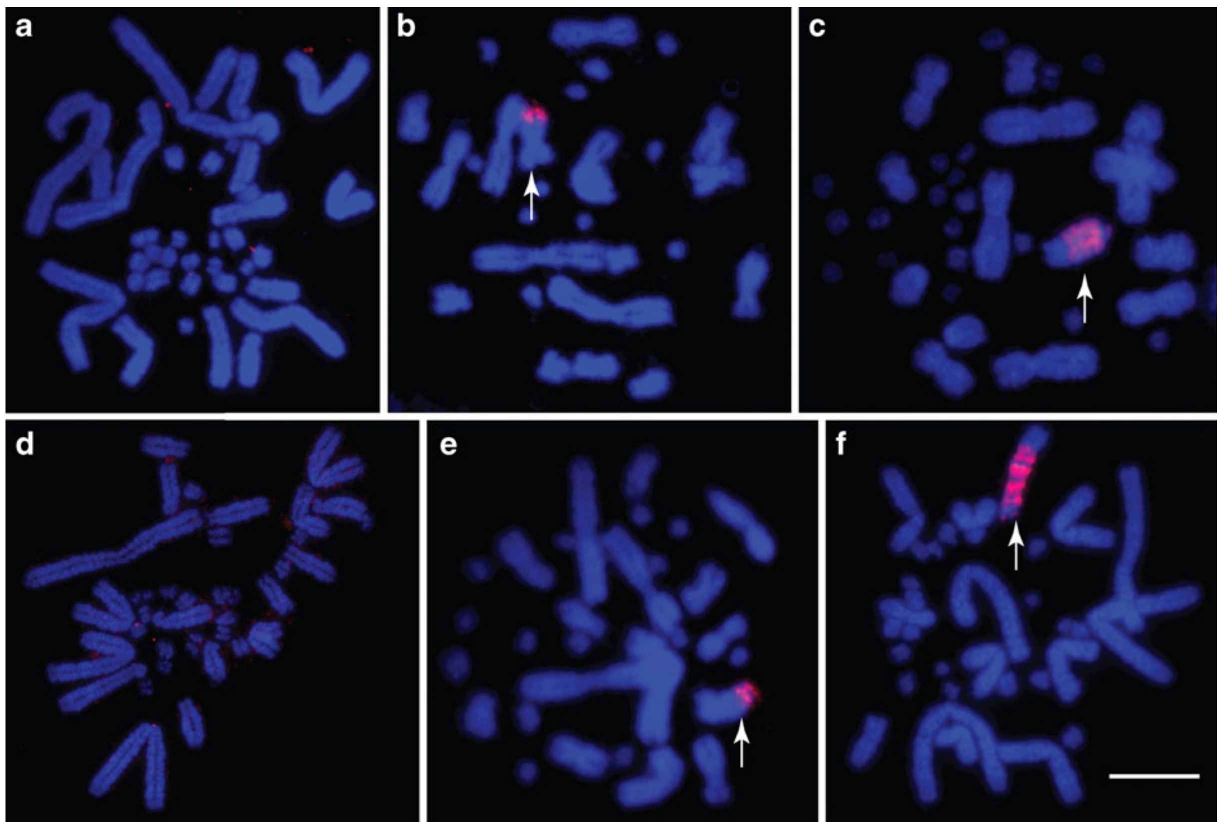

Fig. 2 Hybridization of *Bkm*-related oligonucleotide probes to female metaphase spreads of **a**, **d** *L. fuscus*, **b**, **e** *S. cucullatus*, and **c**, **f** *N. scutatus*. The probe is $(GATA)_4$ in *upper images* and $(GACA)_7$ in *lower images*. *Arrows* indicate the W chromosome, and the *scale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

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).

The *CTNNB1* terminal intron probe also produced an intense signal along the female-specific region of the W but no signal on the Z chromosome, where the gene is located in all snakes so far examined (Fig. 3c). The distal region of the short arm of chromosome 6 has a large heterochromatic block (Mengden 1982) that was also marked by this probe.

We sequenced the cloned 5.4 kb *DMRT1* and 1.2 kb *CTNNB1* introns using a primer walking strategy in an attempt to characterize the W-located 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, the *DMRT1* intron 1 contains some extended mononucleotide stretches. We searched the GenBank nonredundant (nr) database by restricting the query to Serpentes (snakes). The *DMRT1* intron 1 sequence was found to have homology to intronic sequences of *MYH2* and a number of venom genes (85–96% identity over 45–134 bp), and to flanking sequences of microsatellites. No such pattern was observed for the terminal intron of *CTNNB1*; however, when querying the entire nonredundant 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 (20.72 Mb) 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 chromo-

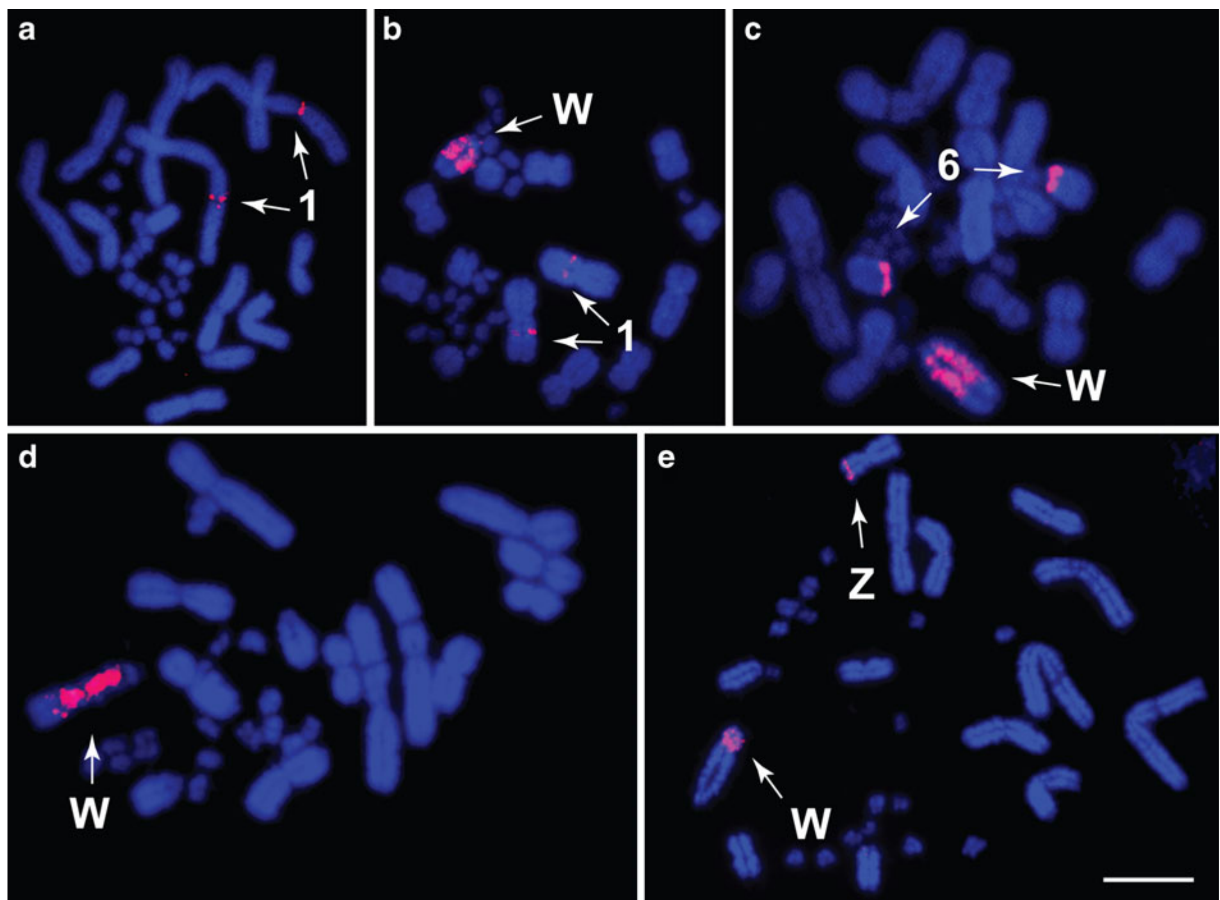

Fig. 3 Heterologous 18S rDNA probe hybridized to *N. scutatus* male (**a**) and female (**b**) metaphase chromosomes. Hybridization of homologous probes containing **c** the terminal intron *CTNNB1*, **d** intron 1 of *DMRT1*, and **e** the terminal intron of *DMRT1* from *N. scutatus* on female metaphase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

somes by examining the location of orthologs in the sequenced genomes of human (HSA) and *X. tropicalis* (XTR; Table 3). First, we looked for ancestral synteny of orthologs from GGA2 and GGA27 to determine the antiquity of the snake Z chromosome. We found that GGA2 shares regions of orthology with HSA3, 7, 8, and 18 (Standardized residuals=10.03, 27.42, 34.17, 32.37, respectively, $p \ll 0.0001$) and GGA27 is equivalent to part of HSA17 (Std Residual=52.55, $p \ll 0.0001$); in *Xenopus*, GGA2=XTR6 (Std Residual=61.87, $p \ll 0.0001$) and GGA27 is equivalent to part of XTR10 (Std Residual=35.29, $p < 0.0001$). In each species, none of the chromosomes orthologous to GGA2 was 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 and *Xenopus* and compared their locations to GGA2 and GGA27 orthologs, as already identified. In humans, GGAZ=HSA5, 9, 18 (Std Residuals=46.02, 44.44, 11.00; $p \ll 0.0001$); and in *Xenopus*, GGAZ is equivalent to part of XTR1 (Std Residual=35.98; $p \ll 0.0001$). On HSA18, 236 chicken–human orthologs are known: 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. Of these 236 orthologs, only one, *SS18*, has been mapped in a snake (to chromosome 3q in *E. quadrivirgata*; Srikulnath et al. 2009), so this should not be considered strong evidence for an ancestral association of chicken and snake Z chromosomes.

Table 3 Contingency table showing the number of orthologs on *X. tropicalis* chromosomes that map to the chromosomes of chicken and human

Xenopus chromosome	"Snake Z"				GGA-XTR	HSA-XTR
	GGA2	GGA27	GGAZ	HSA18	Totals	Totals
XTR1	12	14	372**	44*	1,116	1,466
XTR2	7	2	1	6	577	860
XTR3	0	0	3	5	495	593
XTR4	6	1	4	2	704	877
XTR5	1	0	0	1	535	595
XTR6	750**	2	0	65**	842	900
XTR7	3	1	4	0	486	667
XTR8	1	1	2	2	625	908
XTR9	5	1	2	1	564	682
XTR10	3	64**	0	0	214	293
Totals	788	86	388	126	∑6,158	∑7,841

The snake Z chromosome is orthologous to GGA2 and 27, and in *Xenopus*, these genes also map to separate chromosomes (XTR6 and XTR10), suggesting that their synteny on the snake Z chromosome postdates the divergence of squamates. In humans, some orthologs of GGA2 and GGAZ genes map to HSA18, but they map to different chromosomes in *Xenopus*, as is the case for other snake Z (GGA2 and GGA27) and GGAZ genes, and so ancestral synteny of bird and snake sex chromosomes is not supported. Significance is based on a z test of standardized residuals as described in the text

*p<0.01 **p< <0.0001

Discussion

Repeat accumulation on snake W chromosomes

We mapped the distribution of several repetitive DNA sequences on snake W chromosomes that differ by degree of differentiation. We showed that these snake W chromosomes differ substantially in their pattern of repeat accumulation with increased W chromosome differentiation and that this is accompanied by an increasingly complex suite of repetitive DNA located on the female specific region.

In the water python *L. fuscus*, we found no evidence of any amplified sequences on a W chromosome (Figs. 1a, d and 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 and Stock 1980; Mengden 1982). Nor could we detect any female-specific region of the python W using CGH, which should be sensitive enough to detect differential segments as small as 2 to 3 Mb (Schoumans et al. 2004). This confirms that the differential segment is particularly small in pythons, unless it is masked by differential amplification of repeats common to both sexes. Sex chromosomes are yet to be demonstrated cytologically in this python but are presumed to be the fourth or fifth largest pair, as in many other snakes (Mengden and Stock 1980).

In contrast, differentiated sex chromosomes have previously been characterized by standard cytogenetic techniques in the colubrid and elapid species we studied (Shine and Bull 1977; Mengden 1981, 1982). The W chromosome in *S. cucullatus* differs from the Z in centromere position (the short arm is slightly shorter) and C banding reveals the whole chromosome to be largely heterochromatic (Mengden 1982). We showed female-specific sequences along its length, but concentrated on the distal region of the long arm (Fig. 1b). This same distal region was heavily marked by *Bkm* probes and the chicken W chromosome paint. A similar pattern was observed in *N. scutatus* but in that species, it is the majority of the W chromosome that bears female-specific sequences which include *Bkm*, *DMRT1*-releated, *CTNNB1*-related, 18S-related repeats, and chicken W chromosome sequences. A different hybridization pattern for each probe suggests that each class of repeat is discrete.

Bkm is often, but not always, associated with heterochromatin both in autosomes and degenerated

sex chromosomes (Jones and Singh 1985; Nanda et al. 1991). Our data are consistent 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 and 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 bind *Bkm* specifically have been isolated from the gonads of snakes, silkworms, mice, and man (Singh et al. 1976, 1981; 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. 1976, 1981). The absence of this repeat in large domains on the sex chromosomes of pythons argues against a conserved, functional role for *Bkm*.

Although probes from the first intron of *DMRT1* and the terminal intron of *CTNNB1* produced repetitive patterns on the W, sequence analysis using RepeatMasker did not reveal any known repetitive element. Sequence similarity with introns of unrelated genes suggests that they may contain extinct and now degenerate transposable elements. We suggest that hybridization 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. 3e). This explains why the subtelomeric region of the W chromosome long arm was unmarked by the CGH probe (Fig. 1c). 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 and Steinemann 2005).

The NOR is located on chromosome 1 of *N. scutatus*, as for most elapid snakes, and silver staining shows that this is the only active NOR, at least in somatic tissues (Mengden 1981, 1982). Our study confirmed its autosomal location using FISH, and revealed that 18S-related repeats are also amplified on the W chromosome (Fig. 3a, b). 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 no female was examined to determine whether the W also contains a NOR (Porter et al. 1991). Active NORs have been mapped to both sex chromosomes in the turtle *P. sinensis* (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 as a ladder-like pattern along its length. In the ancestor of macropodid marsupials (kangaroos), the only NOR was translocated to the X and Y, but in most species, the rDNA sequences degraded on the Y and lost their activity (Toder et al. 1997). We suggest that the repetitive nature of the NOR lends itself to amplification on the sex chromosomes of many animal species.

There may be functional significance for amplification of W-borne 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 NOR activity has been made during reptile oogenesis, but W-specific amplification of this locus may facilitate elevated ribosome production. It would be, therefore, interesting to investigate the activity of W-linked ribosomal sequences during oogenesis in these species.

Evolution of snake sex chromosomes

Comparative mapping of genes on sex chromosomes in three species of snakes indicates that W chromosome differentiation 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 *P. molurus* and *P. flavoviridis*. This repeat is found on the short arm of the W of *E. quadrivirgata*, but the long arm of the Z chromosome, suggesting that the centromere has been repositioned. The order of gene loss on the degenerated W chromosome of *E. quadrivirgata* and *P. 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 (O'Meally et al. in prep). Our mapping in the colubrid *S. cucullatus* shows repeats and heterochromatin to be located on the long arm of the W chromosome suggesting, that, like *E. quadrivirgata*, the centromere has been repositioned. 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 sequences. The large size of the W chromosome may indicate a recent addition of repetitive DNA following rearrangement, which is thought to occur shortly after recombination between sex-specific genes 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 and Page 1999; Nicolas et al. 2005).

Whereas the W chromosome is highly variable, gene blocks on the snake Z chromosome are remarkably well conserved, even between snakes and some agamid lizards. For example, six genes that map to the short arm of chromosome 6 in the central bearded dragon *Pogona vitticeps* are located on the short arm of the Z in *E. quadrivirgata* and the short arm of GGA2 (Ezaz et al. 2009b). Likewise, the synteny of four snake Z genes is conserved on chromosome 6 in the butterfly lizard *Leiolepis reevesii rubritaeniata*, with one mapping 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 probably came about after early snakes diverged from other Toxicofera, but before pythons arose (105MYA). Alternatively, the snake Z could have operated as a sex chromosome in a common ancestor but lost that role in agamids. Wider taxonomic sampling is required to test this proposition. In any case, synteny of these genes has been conserved since the divergence of snakes and agamids from a common ancestor, about 166 MYA (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 after the divergence of a common amniote ancestor. Interestingly, it appears that GGA2 and 27 derive from the same ancestral vertebrate chromosome "E" proposed 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.

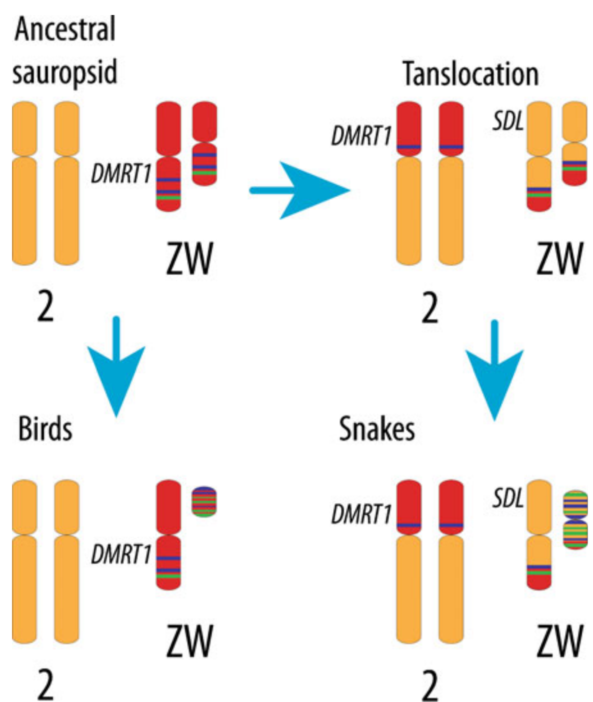

Fig. 4 Did the repeats shared between the sex chromosomes of snakes and birds arise due to a Z-autosome translocation? In this scenario, the ancestral sauropsid had a bird-like ZW system governed by dosage of *DMRT1*. There were repetitive elements (*blue and green lines*) shared by the ZW pair (as observed), but much amplified on the W chromosome. In birds, the ZW further differentiated as the W chromosome degenerated and repeats were amplified. In the snake lineage, there was a translocation between parts of chromosomes 2 and Z. The bulk of the Z became chromosome 2p in snakes. A small region of Z chromosome carrying a few copies of the repeats was translocated to ancestral chromosome 2p, which became the ZW of snakes with the acquisition of a new sex-determining locus *SDL*. The same repeats were amplified on the new snake W chromosome

Implications of sex-chromosome repeats shared between snakes and birds

Hybridization of the chicken W chromosome paint to the W chromosome of derived snakes suggests that repetitive 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 and Voss (2007) on the basis of their synteny in the amphibian *Ambystoma*. We examined the location of orthologs from GGAZ and those from GGA2 and 27 (as a proxy for snake Z genes) in a tetrapod outgroup (*Xenopus*) but found no evidence of large-scale synteny conservation. Within amniotes, 176 genes from GGA2 and 48 from GGAZ are syntenic in HSA18. If this were the arrangement in a common ancestor of snakes and birds, it could be sufficient to explain the shared sex chromosome repeats. The synteny of HSA18 genes, however, is likely to have arisen independently in the mammalian lineage and so represent a derived rather than an ancestral state as implied by the ancestral amniote karyotype proposed by Nakatani et al. (2007) and the arrangement of these genes in *Xenopus* (Table 3). In tuatara, although the map coverage is light, orthologs from snake, bird and mammal sex chromosomes map to different autosomes (O'Meally et al. 2009). An independent rearrangement involving ancestral chromosomes 2 and Z in the lineage leading to snakes (Fig. 4), and amplification of the same repetitive sequences independently on the snake and bird Z chromosomes, cannot yet be discounted, and further data from squamate reptiles are required to test this hypothesis.

Another possibility is that the same repeats arose 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 0.6–0.7 kb unit containing 21 bp tandem repeats organized around different W chromomeres; together they account for 65% of W chromosome DNA and are thought to be involved in heterochromatin assembly (Tone et al. 1984; Kodama et al. 1987; Saitoh and Mizuno 1992; Suka et al. 1993; Solovei et al. 1998). The *Ssp*I family consists of a 0.5 kb unit with a terminal 120 bp tandem repeat that is GA rich, and accounts for about 10% of W chromosome DNA (Itoh and 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 and Mizuno 2002). This suggests that no more than 20–25% (about 10 Mb) of the *Gallus* W is homologous with the W of 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; 167.4 kb) 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 (data not shown).

Without having characterized the shared 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 repeated sequences accumulated independently on the non-homologous snake and bird W chromosomes, as these chromosomes degenerated, and are functionally homologous. Given the large evolutionary distance between birds and snakes, such convergent degeneration could be a general property of sex chromosome differentiation.

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