Sequence and gene content of a large fragment of a lizard sex chromosome and evaluation of candidate sex differentiating gene R-spondin 1

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Abstract

Background: Scant genomic information from non-avian reptile sex chromosomes is available, and for only a few lizards, several snakes and one turtle species, and it represents only a small fraction of the total sex chromosome sequences in these species.

Results: We report a 352 kb of contiguous sequence from the sex chromosome of a squamate reptile, *Pogona vitticeps*, with a ZZ/ZW sex microchromosome system. This contig contains five protein coding genes (*oprd1*, *rcc1*, *znf91*, *znf131*, *znf180*), and major families of repetitive sequences with a high number of copies of LTR and non-LTR retrotransposons, including the CR1 and Box-B LINES. The two genes, *oprd1* and *rcc1* are part of a homologous syntenic block, which is conserved among amniotes. While *oprd1* and *rcc1* have no known function in sex determination or differentiation in amniotes, this homologous syntenic block in mammals and chicken also contains *R-spondin 1* (*rspo1*), the ovarian differentiating gene in mammals. In order to explore the probability that *rspo1* is sex determining in dragon lizards, genomic BAC and cDNA clones were mapped using fluorescence in situ hybridisation. Their location on an autosomal microchromosome pair, not on the ZW sex microchromosomes, eliminates *rspo1* as a candidate sex determining gene in *P. vitticeps*.

Conclusion: Our study has characterized the largest contiguous stretch of physically mapped sex chromosome sequence (352 kb) from a ZZ/ZW lizard species. Although this region represents only a small fraction of the sex chromosomes of *P. vitticeps*, it has revealed several features typically associated with sex chromosomes including the accumulation of large blocks of repetitive sequences.

Keywords: ZW sex chromosomes, Genotypic sex determination (GSD), Temperature dependent sex determination (TSD), RSPO1, Squamata, Reptilia

Background

Chromosomes are remarkably conserved across vertebrates, showing striking conservation of gene content and gene order of substantial regions of synteny over millions of years. For example, the karyotypes of different bird species are extremely similar [1] and comparative chromosome painting of squamate reptiles (lizards and snakes) macrochromosomes shows ancestral homology dating back to the early Jurassic [2,3].

In remarkable contrast to this deep conservation, and surprising, considering their critical role in reproduction, sex chromosomes show a lack of homology and frequent rearrangements and transitions between and even within lineages [4]. The most stable and conserved vertebrate systems are in therian mammals (eutherians and marsupial mammals with a highly conserved XX female: XY male system of male heterogamety), and birds and snakes (with highly conserved ZW female: ZZ male system of female heterogamety). These three chromosome
systems, that is, XY of mammals, ZW of snakes and 
ZW of birds show no genetic homology and are 
thought to have evolved independently [4,5]. The evolu-
tionary dynamism of sex chromosomes is more obvious 
from the distinct forms of XY and ZW chromosomes 
among vertebrates even within taxonomic families [5,6]. 
It is this lability and the role of the sex chromosomes 
in the fundamental trait of sex determination that 
makes non-mammalian vertebrates of extreme interest 
for studying the processes behind genome organization 
and evolution [5-8].

Sex chromosomes are generally thought to have 
evolved from a homologous autosomal pair, when one 
partner acquired a sex determining gene [9-11]. Accumu-
lation of sexually advantageous mutations near the sex 
determining locus led to selection for suppression of re-
combination, which in turn accelerated the loss of active 
genes, deletions and insertions from the sex specific Y or 
W chromosomes [11-14]. The loss of genetic material 
and accumulation of repetitive sequences on the Y or W 
is obvious in many species as cytological heteromorph-
ism. This process of repeat accumulation is rapid and 
and stochastic, generating various intermediates between 
homomorphy and extreme differentiation in squamate 
reptiles (snakes and lizards), and variation in the gene 
content as can be seen in the Y chromosome of different 
mammals [6,15].

Despite the discovery of XY and ZW sex chromosomes 
in the early 19th century [16-18], few master sex deter-
mining genes have been identified. These include SRY in 
therian mammals [19,20], and a variety of genes in fish 
and frog: dmy in medaka (Oryzias latipes) [21,22], gsdf 
(Y) in medaka (O. luzonensis) [23], dmyw in clawed frog 
(Xenopus laevis) [24], sdy in rainbow trout (Oncorhyn-
clus mykiss) [25], and amhy in Patagonian pejerrey 
(Odontesthes hatcheri) [26]. In all but three cases (sdy in 
rainbow trout, amhy in Patagonian pejerrey and gsdf(Y) 
in medaka) [23,25,26], master sex determining genes are 
transcription factors, in so far as they appear to act by 
up- or down-regulating transcription in the vertebrate 
sex differentiation pathway. This newly discovered diver-
sity in regulation suggests that the de novo evolution 
of master sex determining genes from genes that are 
not involved in sex differentiation can occur in verte-
brates. It occurs less frequently than those that are 
involved in the sexual differentiation pathway. How-
ever, due to recent advancement in molecular genetics 
and genome sequencing technologies, novel master sex 
determining genes are likely to be discovered at an acce-
lerating rate, in turn discovering novel mechanisms of sex 
determination.

Reptiles are of particular interest because they display 
avnishing variation in the sex determining system. 
They are represented by species which display all types 
of chromosomal system (e.g. XY, ZW, including multiple 
sex chromosomes) and temperature-dependent sex de-
termination (TSD), as well as species in which the two 
modes of sex determination interact [6,8,27-29]. This 
diversity is well established in the literature, so it is re-
markable that little is known of the genes involved. 
Non-avian reptiles remain the only vertebrate group in 
which no master sex determining gene has been identi-
ified. They therefore promise to yield novel insights into 
vertebrate sex determination.

The best-studied sex chromosomes among non-avian 
reptiles are those of snakes [30-34]. Like birds, snakes 
have a ZZ male ZW female system, and Z gene content 
is conserved across all species so far examined. However, 
gene mapping shows that the sex chromosomes of 
snakes are not homologous to those of birds or mam-
mals [31,32], nor to those of a ZW turtle (Pelodiscus 
sinensis), a ZW gecko (Gekko hokouensis), a ZW dragon 
lizard (Pogona vitticeps) or an XY green anole (Anolis 
carinensis) [31,32,35-38]. Four genes that are Z-linked 
in G. hokouensis are autosomal in the dragon lizard 
Pogona vitticeps [35] and in snakes [32,37] and five 
genomes that are Z-borne in snakes and birds are auto-
somal in the dragon lizard P. vitticeps [35]. Even within 
dragons (Agamidae), comparative mapping of a sex-
linked marker shows that the ZW sex microchromo-
osomes of three Australian agamid species (P. vitticeps, 
P. barbata and Diporiphora nobbi) are homologous, 
but their homology does not extend to a fourth species, 
Ctenophorus fordi [29,39]. Thus there is likely to be a 
number of independent origins of the sex chromosomes, 
not only between two families of lizards (Gekkonidae and 
Agamidae), but also among closely related species in the 
same subfamily.

The central bearded dragon, P. vitticeps, is a powerful 
model for the study of the evolution of sex determina-
tion because it has differentiated sex chromosomes 
[40], but phenotypic sex can be reversed by high tempera-
tures, producing all female individuals [28]. Thus, sex in 
P. vitticeps is determined by the interplay between geno-
type and environment, producing genotype-phenotype 
discordant individuals. Discovering novel sex determining 
genes in this species will therefore broaden our under-
standing of how sex is genetically determined, as well as of 
how environment can regulate genetic machinery. This 
species has 16 pairs of chromosomes – 6 pairs are macro-
chromosomes and 10 pairs are microchromosomes [41] – 
with female heterogamety (ZZ male, ZW female) [40]. 
The sex chromosomes are microchromosomes and the W 
chromosome is larger than the Z [42]. The W chromo-
some is also highly heterochromatic [40] which suggests 
that its sequence content is different from the Z and that 
an amplification of repetitive DNA is likely to have oc-
curred during its evolution.
We previously reported the isolation of Z and W linked markers, developed a genetic sex test and sequenced a 3.2 kb region of the sex chromosomes [28,29] in *Pogona vitticeps*. In this study, we extend the sequenced region of the *P. vitticeps* sex microchromosomes to 352 kb using a BAC walking approach. We report 28 open reading frame (ORFs) including three gene family members (*oprд, rcc* and *znf*), and eight major groups of repetitive elements. Comparative analysis of this partial sex chromosome sequence, allowed us to identify additional potential candidate sex chromosome genes in *P. vitticeps*. One of these genes, *R-spondin 1 (rspo1)*, has a central role in vertebrate ovarian differentiation [43-45] and we evaluate it as a candidate sex-determining gene in *P. vitticeps*.

**Results**

**Isolation and physical mapping of *Pogona vitticeps* sex chromosome BAC clones**

Ten BAC clones were isolated by genomic library screening with previously identified sex linked sequences [27,28] and chromosome walking. We sequenced five of the 10 BAC clones, which ranged in length from 98 kb to 202 kb (GenBank Accessions: KF541650; KF541651; KF541652; KF541653; KF541655, Table 1), while the remaining five BAC clones overlapped the sequenced clones. Reads from all five BAC clones were assembled into a single contiguous sequence of 352 kb (GenBank Accession: KF541658). Analysis of this partial sex chromosome sequence revealed that the contig contains the previously identified W marker Pv72W and contig C1, but not the Z marker Pv71Z [27,28] (Figure 1a). This implies that the contig is likely to have W chromosomal origin and the contig therefore represents partial W sequence (Figure 1a). The BAC clones were physically mapped by DNA FISH as well as by fiber FISH to verify their location on the sex chromosomes (Figure 1b, e). The intensity and pattern of hybridisation signals of two colour DNA FISH with BAC chromosome sequences (Figure 1c and d, Table 1, Additional file 1). For example, one of the smallest sex chromosome BAC clones, Pv03_L07 (about 98 kb) contains 41% repetitive sequences of which 43% are non-LTR retrotransposons (Table 1). Overall, LTR and non-LTR retrotransposons constituted the bulk of the repetitive sequences of the sex chromosome BAC clones. We compared this composition with that of three autosomal BAC contigs. In general, sex chromosome BAC clones have three times more repetitive sequences (Table 1). Autosomal BAC clones showed no preferential accumulation of any specific repetitive sequences (Table 1).

We also investigated the accumulation of microsatellite repeats within the 352 kb sex linked contig using Msat commander [49]. Our analysis identified 16 STR loci consisting of three di-nucleotide (AC, AG, AT), three tri-nucleotide (AAC, AGC, AAT), eight tetra-nucleotide (AAGG, AATC, AATG, ACGC, AAAC, ATCC, AGAT, AAAT), and two penta-nucleotide (AAAAA, AAATC) repeat motifs within the 352 kb sex linked contig. At least five of these loci were found to be amplified on the W chromosomes (data not shown, will be published elsewhere). These loci therefore provide opportunities to develop sex-linked markers.

**Comparative analysis of the sequenced region**

Comparative orthology analysis revealed that orthologues of *oprд1* and *rcc1* are part of a conserved homologous syntenic block (HSBs) in other vertebrates (e.g. chicken, chromosome 23; human chromosome 1). This region is not on the sex chromosomes of these other species, confirming the independent origin of the dragon sex chromosomes.

Analysis of genes in this HSB identified a candidate sex determining gene for *P. vitticeps*, the mammalian
| Repeat class         | Sex chromosome BAC clones |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|----------------------|---------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|                      | Pv03_L07 (98 kb)          | Pv151_P16 (183 Kb) | Pv159_B13 (113 Kb) | Pv237_P23 (202 kb) | Pv151_D05 (156 Kb) |          |          |          |          |          |          |          |          |          |          |          |
|                      | Fragments | Size (bp) | Fragments | Size (bp) | Fragments | Size (bp) | Fragments | Size (bp) | Fragments | Size (bp) | Fragments | Size (bp) | Fragments | Size (bp) | Fragments | Size (bp) |
| Integrated virus     | 0         | 0         | 0         | 0         | 1         | 47        | 1         | 47        | 0         | 0         | 0         | 0         | 0         | 0         | 0         | 0         |
| Interspersed repeat  | 1         | 49        | 3         | 254       | 2         | 126       | 1         | 49        | 1         | 77        | 2         | 165       | 0         | 0         | 0         | 0         |
| DNA transposon       | 30        | 2872      | 53        | 4050      | 37        | 3250      | 69        | 5288      | 58        | 4343      | 58        | 5283      | 49        | 6140      | 40        | 4906      |
| Endogenous retrovirus| 23        | 5252      | 39        | 6836      | 9         | 489       | 16        | 1036      | 11        | 726       | 25        | 1888      | 16        | 1132      | 14        | 990       |
| LTR transposon       | 37        | 12385     | 87        | 25553     | 48        | 9078      | 82        | 12342     | 61        | 5673      | 44        | 3613      | 66        | 4350      | 71        | 5287      |
| Non-LTR transposon   | 58        | 17770     | 101       | 28645     | 74        | 17366     | 148       | 46284     | 121       | 40003     | 46        | 5767      | 51        | 5000      | 54        | 5448      |
| Pseudogene           | 0         | 0         | 0         | 0         | 0         | 0         | 1         | 68        | 1         | 68        | 2         | 173       | 0         | 0         | 0         | 0         |
| Simple repeat        | 21        | 2189      | 35        | 3743      | 6         | 597       | 37        | 3710      | 29        | 2941      | 0         | 0         | 2         | 137       | 2         | 133       |
| Total                | 170       | 40517     | 318       | 69081     | 177       | 30953     | 355       | 68824     | 282       | 53831     | 177       | 16889     | 184       | 16759     | 181       | 16764     |
| Proportion (%)       | 41.3439   | 37.74918  | 27.392    | 34.0713   | 34.5071   | 11.6476   | 13.9658   | 10.4775   | 10.4775   | 10.4775   | 10.4775   | 10.4775   | 10.4775   | 10.4775   | 10.4775   | 10.4775   |
**Figure 1 Isolation and physical mapping of sex chromosome BAC clones in *Pogona vitticeps***. 

**a:** schematic representation of 352 kb partial sex chromosome contig showing relative locations (not to scale) of fully and end sequenced BAC clones, previously identified W-linked contig and AFLP marker, proteins and genes. **b:** two colour FISH showing locations of two sex chromosome BAC clones at either end of the *P. vitticeps* 352 kb contig (Pv151_P16 green and Pv151_D05 orange; yellow denotes overlapping signals). Hybridization signal differences between Z and W chromosomes are visible. **c:** scalable vector graphics (SVG) plot (generated by Repbase-GIRI [48]) of sex chromosome BAC clone Pv03_L07, high density and frequency of red vertical bars represent distributions and locations of repetitive sequences. **d:** SVG plot of autosomal BAC clone Pv176_G09 showing low frequencies of red vertical bars indicating accumulation of low number of repeats compared to that of the sex chromosome BAC clone. **e:** two colour fiber FISH (Pv151_P16 orange, Pv237_P23 green), showing orange and green regions and regions of overlap (yellow). Scale bars represent 10 μm.
ovarian differentiation gene R-spondin 1 (RSPO1). Since, we did not find rspo1 sequences within the sequenced region, we cloned and mapped the dragon rspo1 orthologue.

Cloning and characterization of rspo1
We screened the BAC library using primers designed from sequences obtained from a testis transcriptome (T. Ezaz unpublished). Using this approach we isolated two rspo1-containing BAC clones (Pv100_O13 and Pv215_D15; GenBank Accessions: KF541656; KF541657). These BAC clones were annotated using Genscan [46], revealing 19 and 17 ORFs respectively (Additional file 2). Blastp [47] analysis of those ORFs identified 10 genes from the region surrounding rspo1 in other amniote species; Figure 2, Additional file 2), corresponding to the conserved HSB homologous to chicken chromosome 23 and human chromosome 1.

We also cloned cDNA of a homologue of rspo1 in P. vitticeps by PCR amplification of RNA using primers

| No. | Length (aa) | Predicted genes/proteins | Total score | Query cover | E value | Max identity | Accession anolis |
|-----|-------------|---------------------------|-------------|-------------|---------|--------------|-----------------|
| 1   | 776         | PREDICTED: receptor expression-enhancing protein 3-like | 94.4        | 12%         | 5E-21   | 55%          | XP_003223495.1  |
| 2   | 2208        | PREDICTED: hypothetical protein in LOC100567032 | 302         | 27%         | 3E-82   | 32%          | XP_003229509.1  |
| 3   | 576         | PREDICTED: protein FAM135-B like | 33.5        | 18%         | 0.33    | 26%          | XP_003219535.1  |
| 4   | 718         | PREDICTED: zinc finger protein 91-like | 9847        | 83%         | 0       | 78%          | XP_003220997.1  |
| 5   | 978         | PREDICTED: retrotransposable element Tf2 155 kDa protein type 1-like | 423         | 82%         | 1E-83   | 36%          | XP_003224123.1  |
| 6   | 92          | PREDICTED: leucine-rich repeat and fibronectin type III domain-containing protein | 27.7        | 54%         | 0.65    | 34%          | XP_003222916.1  |
| 7   | 1575        | PREDICTED: retrotransposable element Tf2 155 kDa protein type 1-like | 360         | 40%         | 3E-79   | 39%          | XP_003220874.1  |
| 8   | 340         | PREDICTED: 1, 25-dihydroxvitamin D(3) 24-hydroxylase, mitochondrial-like | 113         | 37%         | 2E-27   | 48%          | XP_003228807.1  |
| 9   | 1092        | PREDICTED: zinc finger protein 135-like | 3327        | 41%         | 0       | 73%          | XP_003229099.1  |
| 10  | 1012        | PREDICTED: hypothetical protein LOC100556889 | 155         | 26%         | 7E-39   | 34%          | XP_003217306.1  |
| 11  | 1009        | PREDICTED: retrotransposable element Tf2 155 kDa protein type 1-like | 364         | 65%         | 7E-81   | 40%          | XP_003220874.1  |
| 12  | 204         | PREDICTED: serologically defined colon cancer antigen 8-like | 57          | 45%         | 9E-10   | 40%          | XP_003225236.1  |
| 13  | 298         | PREDICTED: 1, 25-dihydroxvitamin D(3) 24-hydroxylase, mitochondrial-like | 68.6        | 42%         | 5E-13   | 34%          | XP_003228807.1  |
| 14  | 573         | PREDICTED: hypothetical protein LOC100566709 | 253         | 43%         | 5E-73   | 48%          | XP_003227941.1  |
| 15  | 297         | PREDICTED: RNA-binding protein with multiple splicing-like, partial | 31.2        | 8%          | 0.41    | 56%          | XP_003227406.1  |
| 16  | 418         | PREDICTED: delta-type opioid receptor-like | 623         | 90%         | 0       | 82%          | XP_003229441.1  |
| 17  | 118         | PREDICTED: leucine-rich repeat neuronal protein 2-like | 28.5        | 41%         | 0.94    | 26%          | XP_003220412.1  |
| 18  | 125         | PREDICTED: ubiquitin-conjugating enzyme E2 E1-like isoform 1 | 29.3        | 52%         | 0.42    | 28%          | XP_003226269.1  |
| 19  | 106         | PREDICTED: LOW QUALITY PROTEIN: dynein heavy chain 17, axonemal-like | 28.9        | 81%         | 0.53    | 28%          | XP_003217173.1  |
| 20  | 424         | PREDICTED: regulator of chromosome condensation-like | 794         | 100%        | 0       | 85%          | XP_003229442.1  |
| 21  | 748         | PREDICTED: ras-related protein Rab-17-like | 113         | 18%         | 3E-27   | 44%          | XP_003215266.1  |
| 22  | 57          | PREDICTED: class I histocompatibility antigen, F10 alpha chain-like | 28.1        | 50%         | 0.21    | 31%          | XP_003227795.1  |
| 23  | 527         | PREDICTED: hypothetical protein LOC100561123 | 93.2        | 49%         | 1E-19   | 28%          | XP_003229050.1  |
| 24  | 253         | PREDICTED: excitatory amino acid transporter 2-like | 28.1        | 22%         | 4.5     | 41%          | XP_003226244.1  |
| 25  | 3194        | PREDICTED: hypothetical protein LOC100561123 | 546         | 33%         | 3E-39   | 29%          | XP_003225516.1  |
| 26  | 358         | PREDICTED: ubiquitin carboxyl-terminal hydrolase 25-like | 67.4        | 74%         | 3E-12   | 25%          | XP_003219106.1  |
| 27  | 573         | PREDICTED: hypothetical protein LOC100567032 | 53.9        | 14%         | 2E-07   | 33%          | XP_003229050.1  |
| 28  | 396         | PREDICTED: zinc finger protein 180-like, partial | 1130        | 44%         | 3E-79   | 72%          | XP_003230299.1  |

Only the top hits based on E-values are listed.
designed against *P. vitticeps* testis transcriptome derived *rspo1* (T. Ezaz unpublished). We sequenced three cDNA clones, finding consensus sequences of the two fragments 703 and 956 bp length which partially overlapped. The total length of the consensus sequence covered by the two fragments was 1347 bp.

To eliminate the possibility that *rspo1* gene sequences reside on sex chromosomes in this species, we directly sequenced the PCR products from 10 phenotypic males and 10 phenotypic females. Direct PCR product sequencing was interrupted by the presence of a polymorphic imperfect pentanucleotide repeat (ACCCC	extsubscript{n}) in the intronic region between exon 3 and exon 4 (data not shown). The successfully sequenced regions comprised between 9.6% and 94.4% of the amplicon, although those regions uniformly had a high percent identity with the *rspo1* genomic reference (98.1% - 100% pairwise percent identity) from two fully sequenced BAC clones (Pv100_O13 and Pv215_D15). To circumvent this problem we cloned and sequenced PCR products and confirmed the *rspo1* sequence identity for the full length of the amplicon (Additional file 3).

**Mapping *rspo1***

To determine if *rspo1* lies with *oprd1* and *rcc1* on the sex chromosomes in *P. vitticeps*, we mapped two BAC clones (Pv100_O13 and Pv215_D15) containing *rspo1*. Each BAC clone was physically mapped on to *P. vitticeps* metaphase chromosome spreads along with a *P. vitticeps* sex chromosome specific BAC clone, Pv03_L07 (Figure 3a,b). These two colour FISH experiments (Pv03_L07 + Pv100_O13 and Pv03_L07 + Pv215_D15) showed hybridisation signals on the centromeric region of the W chromosome in females (yellow signal in Figure 3a,b), as well as a single pair of microchromosomes in both males and females (Figure 3a,b, only female patterns are presented in Figure 3).

Since this pattern could have been produced by shared repetitive sequences, we carried out a second round of FISH with the *rspo1* positive BAC clone Pv100_O13, the sex chromosome BAC clone Pv03_L07, and *rspo1* cDNA clone (GenBank Accession: KF541659) on the same chromosome preparations. The cDNA clone mapped only a pair of microchromosomes (Figure 3d). Because of the small size of the cDNA clone (about 1.3 kb), the hybridisation signal strength on chromosomes was weak and the frequency of hybridisation signals per cell was also low. Only about 30% metaphase cells had signals and signals were usually on only one chromosome, which is usually common in FISH mapping of cDNA clones [50]. The autosomal microchromosome to which *rspo1* cDNA clones mapped was identified by co-hybridisation with BAC clone Pv100_O13. No metaphase cell had *rspo1* cDNA signals on sex microchromosomes identified by clone Pv03_L07 (Figure 3e). Thus evidence from the hybridisation of cDNA clone points to *rspo1* residing on an autosomal microchromosome pair and not on the sex chromosomes. In addition, we performed two colour FISH using two *rspo1* BAC clones (Pv215_D15 + Pv100_O13) with male *P. vitticeps* specific c0t-1 DNA as suppressor DNA. Both BAC clones were hybridised onto the same pair of microchromosomes identifying their colocations (Figure 3c). We did not observe any centromeric hybridisation signals on the W chromosomes, suggesting repetitive sequences were successfully suppressed by c0t-1 DNA, which also confirm the cDNA clone mapping, i.e. *rspo1* is autosomal.
Rearrangement of the rcc1-oprd1-rspo1 region in Pogona vitticeps

A closer examination of the region encompassing rcc1, oprd1 and rspo1 indicated that this region was rearranged during amniote evolution. Although the gene content of this region is conserved between human chromosome 1 and chicken chromosome 23, the Genomicus database [51] has placed rcc1 and oprd1 on a putative amniote ancestral block different from that harboring rspo1. A number of different breakpoints are observed in the rcc1/oprd1 region when gene arrangements of the predicted ancestral blocks are compared to human chromosome 1 and chicken chromosome 23 (Figure 4).

The two BAC clones containing rspo1 (Pv100_O13 and Pv215_D15) also contained 10 genes from the region surrounding RSPO1 in other amniote species, but the sequences from these two BACs do not assemble...
well enough to determine whether gene arrangement is also conserved between *P. vitticeps* and other amniotes.

**Discussion**

Our study has characterized the largest contiguous stretch of physically mapped sex chromosome sequence (352 kb) from a ZZ/ZW lizard species. Although this region represents only a small fraction of the sex chromosomes of *P. vitticeps*, it has revealed several features typically associated with sex chromosomes including the accumulation of large blocks of repetitive sequences.

The 352 kb of sex chromosome sequence that we have identified in this study showed large fold accumulation of LTR and non-LTR retrotransposons including CR1, and LINEs (including BovB, L1, L2). Accumulation of such repetitive sequences are common features on sex chromosomes and have been reported for the sex chromosomes of many vertebrate species from insects to mammals [52-60]. In particular, these repeats have been found to be preferentially accumulated on the sex chromosomes with BovB and CR1 repeats found on the mouse Y chromosomes and the chicken Z chromosomes respectively. It appears likely therefore, that these repeats may have converged, preferentially on the sex chromosomes in a number of taxa [33] perhaps acting as an evolutionary driver for the degeneration of sex chromosomes in vertebrates.

Our sequencing and physical mapping strategy revealed two known protein coding genes in addition to three zinc finger genes, within the sequence on the *P. vitticeps* sex chromosomes. The gene *opr1* is a member of G-protein-coupled receptors, which constitute a vast protein family that performs a wide range of functions (including various autocrine, paracrine and endocrine processes) [61,62]. While the gene *rcc1* is a eukaryotic protein which binds to chromatin and interacts with RAN enzyme, a nuclear GTP-binding protein, to promote the loss of bound GTP and the uptake of fresh GTP, thus acting as
a guanine-nucleotide dissociation stimulator (GDS) [63]. The interaction of \textit{rcc1} with RAN probably plays an important role in the regulation of gene expression. Neither of these two genes is known to be involved with sex determination or gonad differentiation of other species and we have no direct evidence that \textit{oprd1} and \textit{rcc1} are involved in the \textit{P. vitticeps} sex determination pathway. Both are expressed in a range of adult mammalian tissues (NCBI UniGene, [64]). Thus these genes, though on the sex chromosomes of \textit{Pogona vitticeps}, are unlikely candidates for a sex determination function.

Orthologues of \textit{oprd1} and \textit{rcc1} are located on a syntenic block that is conserved among amniotes (Figure 4). This synteny suggested an evolutionarily conserved arrangement, which would allow us to identify other genes on the \textit{P. vitticeps} sex chromosomes. One of the genes in this conserved syntenic block, \textit{rspo1}, is involved in ovary differentiation in mammals. This gene was discovered by its mutation in a family with four XX males, and appears to work by activating beta-catenin pathway [43,65]. It was therefore a good candidate for a role in gonad differentiation in \textit{P. vitticeps}. However, it is unlikely to be a candidate for the master sex determining switch as our cDNA clone and two colour BAC FISH mapping has unequivocally established its location on a single pair of autosomal microchromosomes. Although, BAC clones containing \textit{rspo1} sequence mapped to the W chromosome as well as an autosomal microchromosome, this is presumably because they contain repeats shared with the sex chromosomes, which can be suppressed by species specific suppressor DNA (Figure 3c). The absence of \textit{rspo1} from the sex chromosomes eliminates this candidate gene from consideration. Importantly, comparative sequence analysis of this newly characterized \textit{P. vitticeps} partial sex chromosome sequence showed no homology to genes or sequence on other vertebrate sex chromosomes, implying that the ZW sex chromosomes in this species evolved independently [35].

Evolution of the dragon sex chromosomes seems to have involved genome rearrangements (Figure 4). The 352 kb contig contains only two genes \textit{(oprd1 and rcc1)} corresponding to chicken chromosome 23, and they are surrounded by zinc finger genes, which are not present in other amniote species. Intriguingly, \textit{P. vitticeps} is not the only species for which a gene from the ancestral amniote specific syntenic block containing \textit{oprd1} and \textit{rcc1} has ended up on a sex chromosome. For example, \textit{med18}, a gene adjacent to \textit{rcc1} in the predicted ancestral block, is located on the chicken Z chromosome. Is this merely coincidence, or is there a sequence in this region that has a selectable function in sex determination? In contrast, BAC clones containing \textit{rspo1} contain a number of genes found adjacent to this gene in other species. We predict that most of the chicken chromosome 23 syntenic block will reside on the same microchromosome pair in \textit{P. vitticeps} as \textit{rspo1} and not on the sex chromosomes.

**Conclusions**

Our study has demonstrated that the sex chromosomes of \textit{P. vitticeps} share some characteristics of sex chromosomes observed in other vertebrate taxa, namely an accumulation of repetitive elements. However the \textit{P. vitticeps} sex chromosomes also revealed new aspects of sex chromosome evolution that may inform us generally about the rapid evolution of novel sex chromosomes. Of particular interest is our observation that a highly conserved syntenic region that contains \textit{rspo1} was disrupted prior to the formation the \textit{P. vitticeps} sex chromosomes. This work provides the fundamental basis and anchor point from which we can walk to characterize the rest of the dragon W chromosome, and discover a putative sex determining gene(s).

**Methods**

**Isolation and sequencing of BAC clones**

A genomic BAC library (6.2X) for \textit{P. vitticeps} was screened using an overgo-based protocol [35,66]. Overgos were designed against previously identified sex-linked sequences (GenBank Accessions: EU938138.1; EU938139.1; EU938136.1), which were extended from a 50 and a 49 bp W and Z-linked AFLP fragment (Figure 1 for BAC ID) [28,29]. Initial library screening identified five putative sex linked BAC clones. Five more putative sex-linked BAC clones were identified by subsequent BAC walking. We have also used 7-plate PCR matrix of BAC library to isolate BAC clones containing \textit{rspo1}, following manufacturer’s instruction (Amplicon Express, USA). The library was screened using primer pairs (forward: 5’ CAACCTGTGAGCATGTCTTGCAGC 3’; reverse: 5’ AAATCACCTGCAGGATCCAC 3’) designed against a testis transcriptome (T. Ezaz unpublished). Primers were designed to amplify a short genomic fragment between \textit{rspo1} exons 4 and 5 of this gene in \textit{P. vitticeps}. The PCR conditions was as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 90 s; and finally 72°C for 10 min for a final extension.

The full BAC sequences and BAC end sequences were obtained through a commercial vendor (Macrogen, Korea) and Biomorphic Resource Facility (Australian National University, Canberra, Australia) using Sanger and 454 sequencing platforms. In total five sex chromosome BAC clones and three autosomal BAC clones were sequenced (Table 1) using either Sanger or 454 platform. BAC clones were also subjected to end sequencing using pCC1 epicenter vector specific forward and reverse primers: pCC1™ / pgEpFOS™ forward sequencing primer (5’ GGATGTGCTGCAAGGCGATTAAGTT
GG 3’) and pCC1™/pEpiFOS™ reverse sequencing Primer (5’-CTCTGATGTGTGTGGAAATGTGAGC 3’; Invitrogen). We assembled each BAC using the default parameters of Newbler (V2.6, Roche CT, USA), with an expected coverage of 40X and a custom filtering data-base that contained the BAC vector and host genome (DH10B). Sequences shared by the individually assem-bled BAC clones indicated that they were contiguous, so we repeated the assembly with reads from all five BAC clones and mapped reads back to the resulting 352 kb contig. Reads from each BAC occupied a discrete segment of the contig in proportion to the size of the insert, as de-termined by individual BAC assemblies. Sequences were annotated using Genscan [46] and predicted ORFs then subjected to homology search using Blastp (refseq A. caro-linensis [47]). The 352 kb contig was also analyzed for homology using Blastp (nr).

Fluorescence in situ hybridisation (FISH)

BAC clone FISH

The chromosomal locations of isolated sex chromosomal BAC clones were verified by physical mapping using FISH following protocols described in Ezaz et al. [35]. Briefly, 0.5-1 μg of BAC DNA was labeled by nick translation incor-porating directly labeled orange or green dUTP (Abbott Molecular, Botany, NSW, Australia). The labeled BAC DNA was ethanol precipitated, resuspended in 20 μl hybridisation buffer (50% formamide, 10% dextran sulfate, 2X SSC, 40 mmol/L sodium phosphate pH7.0 and 1X Denhardt’s solution), denatured at 68.5°C and hybridised onto metaphase chromosomes spreads overnight at 37°C. Slide washing and microscopy was performed following the protocol described previously by Ezaz et al. [35].

Fiber FISH

Fiber FISH was performed following a protocol described by Heng et al. [67] with modifications to further understand the repeat contents of these BAC clones as well as their arrangements on the sex chromosomes. Briefly, chromatin fiber was obtained by treatment with lysis buffer (0.5% SDS, 50 mM EDTA, 200 mM Tris–HCl pH 7.4). The cell suspension was dropped on slides and air-dried. Slides were soaked in lysis buffer at 90 – 120 s and quickly lifted out to stretch the chromatin fibers and air-dried. After air-drying, the slides were fixed and dehydrated through an ethanol series. The slides were denatured by treatment with 70% 0.1 N NaOH, EtOH 30% (v/v) for 90 s at room temperature. Probe hybridisation, detection and slide analysis was performed following the standard FISH protocol described above.

cDNA clone FISH

We cloned the cDNA of a homologue of rspo1 in P. vit-ticeps. Total RNA was extracted from testicular tissue using RNasy Plus Universal Mini Kit (Qiagen). The cDNA was synthesized by RT-PCR using Oligo (dT)20 Primer and SuperScript III First-Strand Synthesis System (Invitrogen, Australia), and was used as the PCR template to amplify the homologue of rspo1. Two cDNA fragments of the gene were amplified by PCR with the following primer pairs designed against a P. vitticeps’ tes-tes transcriptome: F1, 5’-AGACAAAGCAAGCCAGCAA AC-3’; R1, 5’-GGACAAAGGTTAAGCAGA-3’; F2, 5’-CATCCTAGGAGCCAGGTCTATGTTGTGGAATTGTGAGC 3’; R2, 5’-CTGGC CACGTCTTTACTG G-3’. The PCR conditions was as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; and finally 72°C for 10 min for a final exten-sion. The PCR products were cloned using TOPO TA Cloning Kit (Invitrogen). We sequenced three cDNA clones for respective fragments from a commercial vendor (Macrogen, Korea).

FISH for cDNA clones was performed as described previously by Matsuda et al. [50] with slight modification. The DNA probes were labeled using nick translation kit (Roche Diagnostics) incorporating biotin-16-DUTP accord-ing to standard protocol. Labeled probes were purified by ethanol precipitation, mixed with hybridisation buffer (50% formamide, 2X SSC, 10% dextran sulfate, 1 mg/ml BSA) and denatured by incubation at 75°C for 10 min. Chromo-some slides were denatured in 70% formamide (v/v)/2X SSC for 2 min at 70°C. Approximately, 250 ng of labeled probe (20 μl) per slide was hybridised onto metaphase chromosomes for 1 day at 37°C. Post-hybridisation washes were carried out as following: 50% formamide (v/v)/2X SSC for 20 min at 37°C, 2X SSC for 15 min at room temperature, 1X SSC for 15 min at room temperature and 4X SSC for 5 min at room temperature. The hybridised cDNA probes were reacted with goat anti-biotin antibodies (Vector Laboratories) diluted in 1% BSA/4X SSC for 1 h at 37°C. The chromosomal slides were washed as follows: 4X SSC for 5 min at room temperature, 0.1% IGEPAL/4X SSC for 5 min at room temperature, and 4X SSC for 5 min at room temperature. Then the hybridised probes were reacted with Alexa®888 rabbit anti-goat IgG (Invitrogen). The chromosome slides were washed using the following series; 4X SSC for 10 min at room temperature, 0.1% IGEPAL/ 4X SSC for 10 min at room temperature, and 4X SSC for 10 min at room temperature. The slides were counter-stained with 20 μg/ml DAPI in 2X SSC and mounted with VectaShield (Vector Laboratories).

PCR amplification, cloning and sequencing of rspo1 from multiple Pogona vitticeps individuals

To determine the level of polymorphisms at the se-quence level between male and female P. vitticeps, we screened 20 individuals (10 phenotypic males and 10 phenotypic females). We used the same primer pair to
screen the BAC library and followed the same PCR condition. We cloned eight PCR fragments from three males and three females using pGEM-T Easy vector Systems, (Promega) and TOP10 competent cells (Invitrogen). We sequenced the PCR products using M13 and M13R primers using a commercial vendor (Macrogen, Korea).

Availability of supporting data
All the supporting data are included as additional files.

Additional files

**Additional file 1: Gene content of three autosomal BAC clones.** Sequences were annotated using GenScan [46] and homology search was performed using Blastp [47].

**Additional file 2: Scalable vector graphics (SVG) plot of five sex chromosome and three autosome BAC clones generated by Repbase [48] showing the distribution of repetitive sequences.** This figure highlights high frequency of red vertical bars representing locations of repetitive sequences in sex microchromosomes compared to low frequencies of red vertical bars on autosomes, which are also microchromosomes.

**Additional file 3: Sequence alignment of cloned PCR amplified rsps\(^\circ\) fragments from three males and females and two BAC clones containing rsps\(^\circ\); showing the location of primers and occasional SNPs which are not sex-linked.

Abbreviations

TSD: Temperature-dependent sex determination; GSD: Genotypic sex determination; ORF: Open reading frame; Overgo: Overlapping oligonucleotide probe; BAC: Bacterial artificial chromosome; FISH: Fluorescence in situ hybridisation; Contig: Contiguous assembled sequence; cDNA: Complementary DNA; AFLP: Amplified fragment length polymorphism; SNP: Single nucleotide polymorphism; LINE: Long interspersed nucleic acid element; CR1: Chicken repeat 1 (LINE); GTP: Guanosine-5′-triphosphate; GDS: Guanine-nucleotide dissociation stimulator; BLAST: Basic Local Alignment Search Tool; STR: short tandem repeat (microsatellite).

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

The authors have made the following declaration about their contributions: Conceived, designed and directed the experiments: TE, SDS, AG, JMG. Performed the experiments: TE, DOM, MY, BA, KM, XZ, MJE. Contributed analysis tools: TE, AG, DOM, JD, CH; Wrote the paper: TE, JMG, AG, JD, SDS. All coauthors contributed in reviewing the paper. All authors read and approved the final manuscript.

Funding

This work was funded by ARC Discovery Grants awarded to SDS, AG and SVE (DP0881196), and to TE, SDS, AG, JMG and Yoichi Matsuda (DP110102626). TE and JD are supported by ARC Future Fellowships (FT110100733, FT100100241). We would like to thank Jacqui Richardson and Alistair Zealey for their care of captive animals and Anna MacDonald for initial discussion about microsatellite mining. All experiments were performed with the approval of the University of Canberra Animal Experimentation Ethics Committee.

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Received: 19 August 2013 Accepted: 13 December 2013
Published: 17 December 2013

References

1. Takagi N, Sasaki M: A phylogenetic study of bird karyotypes. Chromosoma 1974, 46:91–120.
2. Pokorna M, Giovannotti M, Kratovil L, Kasai F, Trifonov VA, O’Brien PC, Caputo V, Olmo E, Ferguson-Smith MA, Rens W: Strong conservation of the bird Z chromosome in reptilian genomes is revealed by comparative painting despite 275 million years divergence. Chromosoma 2011, 120:455–468.
3. Pokorna M, Giovannotti M, Kratovil L, Caputo V, Olmo E, Ferguson-Smith MA, Rens W: Conservation of chromosomes syntenic with avian autosomes in squamate reptiles revealed by comparative chromosome painting. Chromosoma 2012, 121:409–418.
4. Ezaz T, Stoglic R, Veyrunes F, Graves JAM: Relationships between vertebrate ZW and XY sex chromosome systems. Curr Biol 2006, 16:736–743.
5. Sarre SD, Ezaz T, Georges A: Transitions between sex-determining systems in reptiles and amphibians. Annu Rev Genomics Hum Genet 2011, 12:391–406.
6. Ezaz T, Sarre SD, O’Meally D, Graves JA, Georges A: Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 2009, 127:249–260.
7. Janes DE, Organ CL, Edwards SV: Variability in sex-determining mechanisms influences genome complexity in reptilia. Cytogenet Genome Res 2009, 127:242–248.
8. Janes DE, Organ CL, Fujita MK, Shedlock AM, Edwards SV: Genome evolution in Reptilia, the sister group of mammals. Annu Rev Genomics Hum Genet 2010, 11:239–264.
9. Fisher RA: The Evolution of Dominance. Biol Rev 1931, 6:345–368.
10. Charlesworth B: The evolution of sex chromosomes. Science 1991, 251:1030–1033.
11. Charlesworth B, Larkins B: The degeneration of \( Y \) chromosomes. Philos Trans B 2000, 355:1563–1572.
12. Nei M: Linkage modifications and sex difference in recombination. Genetics 1969, 63:616–699.
13. Bull JJ: Evolution of sex determining mechanisms. Menlo Park, California: Benjamin/Cummings; 1983.
14. Rice WR: Evolution of the Y sex chromosome in animals. Bioscience 1996, 46:331–343.
15. Graves JAM: Sex chromosome specialization and degeneration in mammals. Cell 2006, 124:901–914.
16. Henking H: Über Patagonian pejerrey (Odontesthes hatcheri) spermatogenese und deren Beziehung zur Entwicklung bei Pyrrhocoris apterus L. Zeitschrift für wissenschaftliche Zoologie 1891, 51:685–736.
17. Stevens NM: Studies in spermatogenesis, with especial reference to the “accessory chromosome”. Carnegie Inst Wash 1905, 36:33.
18. Wilson EB: The Chromosomes in Relation to the Determination of Sex in Insects. Science 1905, 22:500–502.
19. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN: A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 1990, 346:240–244.
20. Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R: Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 1990, 348:450–452.
21. Matsuda M, Nagahama Y, Shinomyya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, et al: DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 2002, 417:559–563.
22. Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, et al: A duplicated copy of DMR1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proc Natl Acad Sci USA 2002, 99:11778–11783.
23. Myoshio T, Otake H, Matsumiya H, Matsuda M, Kuroki Y, Fujimura A, Naruse K, Hanauchi S, Sakazumi M: Tracing the emergence of a novel sex-determining gene in medaka, Oryzias latipes. Genetics 2012, 191:163–170.
24. Yoshimoto S, Ito M: A ZZ/ZZ-type sex determination in Xenopus laevis. FEBS J 2011, 278:1020–1026.
25. Yano A, Guyomard R, Nicolas B, Jouanno E, Quillet E, Klopp C, Cabau C, Bouchez O, Fostier A, Guignen Y: An immune-related gene evolved into
the master sex-determining gene in rainbow trout, Oncorhyncus mykiss. Curr Biol 2012, 22:1423–1428.

26. Hattori RS, Mural Y, Oura M, Masuda S, Mahi SK, Sakamoto T, Fernando J, Somoza GM, Yokota M, Strussmann CA: A linked-anti-Mullerian hormone duplication takes over a critical role in sex determination. Proc Natl Acad Sci USA 2012, 109:2955–2959.

27. Rodder RS, Quinn AE, Georges A, Sarre SD, Shine R: Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard, Biol Lett 2008, 4:176–178.

28. Quinn AE, Georges A, Sarre SD, Guarni F, Ezaz T, Graves JA: Temperature sex reversal implies sex gene dosage in a reptile. Science 2007, 316:411.

29. Quinn AE, Ezaz T, Sarre SD, Graves JA, Georges A: Extension, single-locus conversion and physical mapping of sex chromosome sequences identify the Z microchromosome and pseudo-autosomal region in a dragon lizard, Pogona vitticeps. Heredity 2010, 104:410–417.

30. Ohno S: Sex Chromosomes and Sex-Linked Genes. Heidelberg, Germany: Springer-Verlag; 1967.

31. Matsuoka Y, Nishida-Umehara C, Tarui H, Kurokawa A, Yamada K, Ioibe T, Ando J, Fujwara A, Hira Y, Nishimura O, et al: Highly conserved linkage homology between birds and turtles: bird and turtle chromosomes are precise counterparts of each other. Chromosome Res 2005, 13:601–615.

32. Matsuoka T, Harui H, Toriba M, Yamada K, Nishida-Umehara C, Agata K, Matsuoka Y: Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. Proc Natl Acad Sci USA 2006, 103:18190–18195.

33. O’Meally D, Patel HR, Stiglec R, Sarre SD, Georges A, Marshall Graves JA, Ezaz T: Non-homologous sex chromosomes of birds and snakes share repetitive sequences. Chromosome Res 2010, 18:787–800.

34. O’Meally D, Ezaz T, Georges A, Sarre SD, Graves JA: Are some chromosomes particularly good at sex? Insights from amniotes. Chromosome Res 2012, 20:7–19.

35. Ezaz T, Moritz B, Waters P, Marshall Graves JA, Georges A, Sarre SD: The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. Chromosome Res 2009, 17:965–973.

36. Kawagushi T, Uno Y, Matushika K, Matsuoka Y, Nishida C: The ZW micro-sex chromosomes of the Chinese soft-shelled turtle (Pelodiscus sinensis, Triodychidae, Testudines) have the same origin as chicken chromosome 15. Cytogen Genome Res 2009, 125:125–131.

37. Kawai A, Ishijima J, Nishida C, Kosaka A, Ota H, Kohno S, Matsuoka Y: The ZW sex chromosomes of Gekko hokusouensis (Gekkonidae, Squamata) represent highly conserved homology with those of avian species. Chromosoma 2009, 118(1):43–51.

38. Alfoldi J, Di Palma F, Grabheir M, Williams C, Kong L, Mauceli E, Russell P, Lowe CB, Glor RE, Jaffe JD, et al: The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature 2011, 477:267–271.

39. Ezaz T, Quinn AE, Sarre SD, O’Meally D, Georges A, Graves JA: Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. Chromosome Res 2009, 17:91–98.

40. Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A, Marshall Graves JA: The lizard genome Pogona vitticeps has Z/W micro-sex chromosomes. Chromosome Res 2005, 13:763–776.

41. Witten JJ: Some karyotypes of Australian agamids (Reptilia: Lacertilia). Austral J Zool 1983, 31:533–540.

42. Young MJ, O’Meally D, Sarre SD, Georges A, Ezaz T: Molecular cytogenetic map of the central bearded dragon, Pogona vitticeps (Squamata: Agamidae). Chromosome Res 2013, 21:361–374.

43. Parma P, Raci O, Vidal V, Chabosiier MC, Dellambique L, Valentini S, Guerra L, Scheff A, Carneiro G: R-spondin is essential in sex determination, skin differentiation and malignancy. Nature Genet 2006, 38:1304–1309.

44. Chassot AA, Ranc F, Gregoire EP, Reepers-Gajadien HL, Takeito MM, Carneiro G, de Rocoj DG, Scheff A, Chabosiier MC: Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. Hum Mol Genet 2008, 17:1264–1277.

45. Zhou L, Charikar P, Ju X, Wu L, Liu G, Mohapatra S, Wang D, Nagahama Y: R-spondins are involved in the ovarian differentiation in a teleost, medaka (Oryzias latipes), BMC Dev Biol 2012, 12:56.

46. Burge C, Karlin S: Prediction of complete gene structures in human genomic DNA. J Mol Biol 1997, 268:78–94.

47. Gish W, States DJ: Identification of protein coding regions by database similarity search. Nature Genet 1993, 3:266–272.

48. Jurka J, Kapitonov VV, Pavlicek A, Klonowski P, Kohany O, Walichwicz J: Repbase update, a database of eukaryotic repetitive elements. Cytogenet Genome Res 2005, 110:462–467.

49. Faircloth BC: msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol Ecol Res 2008, 8:89–94.

50. Matsuda Y, Chapman VM: Application of fluorescence in situ hybridisation in genome analysis of the mouse. Electrophoresis 1995, 16:261–272.

51. Louis A, Muffato M, Roest Crollius H: Genomicus: five genome browsers for comparative genomics in eukaryota. Nucleic acids Res 2013, 41:7070–7075.

52. Ferreira IA, Martins C: Physical chromosome mapping of repetitive DNA sequences in Nile tilapia Oreochromis niloticus: evidences for a differential distribution of repetitive elements in the sex chromosomes. Micron 2008, 39:411–418.

53. Harvey SC, Boochneshke C, Campos-Ramos R, Ezaz MT, Griffin DK, Broman NR, Penman P: Analysis of repetitive DNA sequences in the sex chromosomes of Oreochromis niloticus. Cytogenet Genome Res 2003, 101:314–319.

54. Kraemer C, Schmidt ER: The sex determining region of Chironomus thummi is associated with highly repetitive DNA and transposable elements. Chromosoma 1993, 102:553–562.

55. Cioffi MB, Camacho JP, Bontollo LA: Repetitive DNAs and differentiation of sex chromosomes in neotropical fishes. Cytogenet Genome Res 2011, 132:188–194.

56. Hughes JF, Skaltsky H, Brown LG, Pikitikova T, Graves T, Fulton RS, Dugan S, Ding Y, Buhay CJ, Krenzlik C, et al: Strct evolutionary conservation followed rapid gene loss on human and rhesus Y chromosomes. Nature 2012, 483:88–96.

57. Charlesworth B: Model for evolution of Y chromosomes and dosage compensation. Proc Natl Acad Sci USA 1975, 72:5618–5622.

58. Erlandsson R, Wilson JR, Paabo S: Sex chromosome transposable element accumulation and male-driven substitutional evolution in humans. Mol Biol Evol 2000, 17:804–812.

59. Shedlock AM: Exploring frontiers in the DNA landscape: an introduction to the symposium "Genome Analysis and the Molecular Systematics of Retroelements", Systematic Biol 2006, 55:871–874.

60. Shedlock AM, Botka CW, Zhao S, Shetty J, Zhang T, Liu JS, Deschavanne PJ, Edwards SV: Phylogenomics of nonavian reptiles and the structure of the ancestral amniote genome. Proc Natl Acad Sci USA 2007, 104:2767–2772.

61. Keiffer BL, Befort K, Gavieraux-Ruff C, Hirth CG: The delta-opiod receptor: isolation of a CDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 1992, 89:12048–12052.

62. Stevens CW: The evolution of vertebrate opioid receptors. Front biosci 2004, 14:1247–1269.

63. Malde RD, England JR, Yennawar HP, Tan S: Structure of RCL1, chomatrin factor bound to the nucleosome core particle. Nature 2010, 467:562–566.

64. Schuler GD: Pieces of the puzzle: expressed sequence tags and the catalog of human genes. J Mol Med 1997, 75:694–698.

65. Kim KA, Zhao J, Andarmani S, Kakitani M, Oshima T, Binnerts ME, Abo A, Tomizuka K, Funk WD: R-spondin proteins: a novel link to beta-catenin activation. Cell Cycle 2006, 5:23–26.

66. Ross M, LaBrie T, McPherson S, Stanton VP: Screening large-insert libraries by hybridization. In Current Protocols in Human Genetics. New York: John Wiley Sons; 1999:5.6.1–5.6.32.

67. Heng HH, Squire J, Tsui LC: High-resolution mapping of mammalian genes by in situ hybridisation to free chromatin. Proc Nat Acad Sci USA 1990, 89:9509–9513.

Ezaz et al. BMC Genomics 2013, 14:899 doi:10.1186/1471-2164-14-899

Cite this article as: Ezaz et al.: Sequence and gene content of a large fragment of a lizard sex chromosome and evaluation of candidate sex differentiating gene R-spondin. 1. BMC Genomics 2013 14:899.