



Symposium Article

Origin of Amniote Sex Chromosomes: An Ancestral Super-Sex Chromosome, or Common Requirements?

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Received March 21, 2016; First decision May 9, 2016; Accepted August 22, 2016.

Corresponding editor: Catherine Peichel

Abstract

The diversity of sex chromosomes among amniotes is the product of independent evolution of different systems in different lineages, defined by novel sex-determining genes. Convergent evolution is very common, suggesting that some genes are particularly adept at taking on a sex-determining role. Comparative gene mapping, and more recently whole genome sequencing, have now turned up other surprising relationships; different regions of the amniote genome that have become sex determining in some taxa seem to share synteny, or share sequence, in others. Is this, after all, evidence that these regions were once linked in a super-sex chromosome that underwent multiple fission in different ways in different amniote lineages? Or does it signify that special properties of sex chromosomes (paucity of active genes, low recombination, epigenetic regulation to achieve dosage compensation) predispose particular chromosomes to a sex-determining role?

Subject area: Genomics and gene mapping

Key words: comparative gene mapping, sex chromosome evolution, sex determination

Sex chromosomes are the most dynamic entity in any genome, displaying unique morphology, gene content, and evolution (Muller 1914; Ohno 1967; Charlesworth 1991; Graves 2006; Ezaz and Deakin 2014). The diversity of sex chromosome morphologies among amniotes is truly remarkable, with sex chromosomes ranging from cryptic to highly heteromorphic. Systems include XY (male heterogamety) and ZW (female heterogamety) in which the sex-specific element (Y or W) is a more or less degraded version of the X or Z and are shorter because of deletion or longer because of insertion and amplification.

This diversity is surprising, because a gene on one of the sex chromosomes must trigger the first step in a highly conserved pathway toward sex determination. Advances in DNA technology have

enabled the identification of sex-determining genes in several amniotes, including species of mammals, birds, fish, and frogs. It is clear that many different genes can accomplish the same step; so too can environmental triggers such as temperature, which act through epigenetic regulation of these and other genes.

Despite this diversity, the same few genes have been identified as sex determining in widely divergent amniotes as well as frogs and fish; for instance, *DMRT1* in birds, frogs, and fish, *SOX3* in therian mammals frogs and fish, *AMH* or its receptor in monotremes and different fish species (for review, see Graves 2013). They cannot all be identical by descent. This implies that some genes are innately good at the job of sex determination and are independently recruited to this role in many lineages (Graves and Peichel 2010; O'Meally et al. 2012).

Comparative gene mapping, and more recent whole genome sequencing, have now turned up other surprising relationships; different regions of the amniote genome that have become sex determining in some taxa seem to share synteny, or share sequence, in others. Is there, after all, evidence that these regions were once linked in a super-sex chromosome that was broken up in different ways in different amniote lineages, as was originally suggested for the vertebrate genome (Smith and Voss 2007)? Here, we present data that suggest shared synteny, speculate on its significance, and present alternative explanations.

Sex Chromosome Evolution

How sex chromosomes evolve and degenerate has been a topic for debate for more than a century. Some of the earliest ideas came from studies of sex determination in *Drosophila* and plants, but the principles appear to hold generally for all animals.

Sex chromosomes are proposed to have evolved from an autosomal pair, when one chromosome acquired a sex-determining locus (Muller 1914; Ohno 1967; Charlesworth 1991; Graves 2006). The acquisition of a second sexually antagonistic gene was proposed to drive selection for the suppression of recombination (Charlesworth 1991). A more general hypothesis is that the acquisition of a sex-specific function by any gene linked to the sex-determining locus could have this effect (Rice 1984; Graves 2006).

There are several mechanisms by which loss of recombination between the proto-sex chromosomes leads to inactivation and loss of genes from the sex-specific chromosome (Y or W). These permit large deletions, insertions, inversions, and amplifications in that chromosome, resulting in the evolution of heteromorphic X and Y or Z and W chromosomes.

In all therian mammals, the Y chromosome has been almost terminally reduced and heterochromatinized, so the process is not as evident as it is in some reptiles with younger sex chromosomes. For instance, minimally differentiated XY chromosomes are found in 3 cryptodiran turtles, whose Y chromosomes are smaller than X chromosomes due to a difference in the copy number of 18S–28S rRNA genes but there has been no loss of single-copy functional genes or accumulation of heterochromatin (Kawagoshi et al. 2012, 2014). Accumulation of different microsatellite motifs to the same ancestral W is found in the more differentiated sex chromosomes of Australian varanid lizards, which results from rapid and independent amplification of repeat sequences on W chromosomes (Matsubara et al. 2014b).

Different stages of W chromosome differentiation can also be seen in birds, which have a highly conserved Z but a W that ranges from near equivalence in the ancient palaeognathous birds to highly reduced in other lineages. Little heterochromatin is present in the W chromosomes of ratites, but some accumulation of repetitive DNA sequences was observed on the smaller W chromosome in the elegant crested tinamou (Nishida-Umehara et al. 2007; Tsuda et al. 2007), and the highly differentiated W of neognathous birds is full of repetitive sequences of various origins. In snakes, the W chromosome is often longer than the Z, and full of highly amplified repeats (Matsubara et al. 2006; Olmo 2013; Vicoso et al. 2013; Matsubara et al. 2014b). Comparative analysis of avian and serpent sex chromosome sequences identified different evolutionary origin of snake and bird sex chromosomes as well as different evolutionary strata present in different species within these groups (Matsuda et al. 2005; Vicoso et al. 2013; Zhou et al. 2014).

Relationships Between Amniote Sex Chromosomes

Amniotes comprise mammals and reptiles (including birds). Sauropsida (birds and reptiles) diverged from Synapsida (mammals) around 320 million years ago (MYA) (Shedlock and Edwards 2009). As outlined above, amniotes offer a dazzling array of sex-determining genes and sex chromosomes, as well as many examples of temperature-dependent sex determination (TSD).

Sex Chromosomes of Therian Mammals

There are 3 major groups of extant mammals: eutherians (placental), marsupials, and the egg-laying monotremes. Eutherians diverged from marsupials 166 MYA, and their therian ancestor diverged from the monotremes 190 MYA (Luo et al. 2011). Eutherians have morphologically differentiated XX/XY sex chromosomes with an X highly conserved in size and gene content. The Y chromosome is small, heterochromatic and highly differentiated, as a result of independent degradation in different lineages (Graves 2006; Bellott et al. 2014; Cortez et al. 2014). The mammal sex-determining gene is *SRY* (Sinclair et al. 1990), which evolved from the highly conserved *SOX3* gene, a copy of which remains on the X (Foster and Graves 1994; Graves 2013).

Marsupials also have an XY system, but the marsupial X chromosome shares homology only with the long arm and proximal short arm of the human X chromosome (Graves 1995), defining an ancient X-conserved region (XCR). The short arm of the human X corresponds to a marsupial autosome (Wilcox et al. 1996) and is termed the X-added region (XAR). This implies that chromosome fusion occurred after the divergence of marsupials and eutherians and before the eutherian radiation.

The marsupial Y chromosome shares homology with a segment of the eutherian Y chromosome known as the Y-conserved region, but it also contains a Y-added region, derived from XAR, which constitutes the great majority of the eutherian Y (Waters et al. 2001). The marsupial Y shares 4 genes with the human Y but also contains many active genes that have partners on the conserved XCR, so were presumably lost in eutherians (Pask et al. 2000; Murtagh et al. 2012).

Monotreme Sex Chromosomes

Remarkably, monotremes (platypus and echidnas) have a multiple XY sex chromosome system bearing no relationship with the therian XY pair. Platypus males have 10 unpaired chromosomes ($X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, X_5Y_5$), which form a decavalent chain (Rens et al. 2004). Similarly, the echidnas have 5 X and 4 Y chromosomes, 4 XY pairs sharing homology with the platypus but the fifth with a platypus autosome. This chain formed as a result of serial translocation of an XY pair with autosomes; 3 translocation events in the common ancestor and a fourth independent translocation involving different autosomes after the divergence of platypus and echidna (Rens et al. 2007).

Comparative gene mapping revealed that none of the platypus X chromosomes correspond to the therian X chromosome. Instead, the XCR is homologous to platypus chromosome 6 (Waters et al. 2005; Veyrunes et al. 2008), which also contains *SOX3*, the ancestor of *SRY* (Wallis et al. 2007). This is consistent with the hypothesis that therian sex chromosomes arose from an autosome after therians diverged from monotremes. Notably, the platypus X_5 chromosome largely corresponds to the chicken Z chromosome and appears to be the oldest platypus sex pair (Cortez et al. 2014) (Figure 1). Partial homology with the chicken Z chromosome is also found in $X_{3,q}, X_{2,p}$, and $X_{1,p}/Y_{1,q}$ (Rens et al. 2007; Veyrunes et al. 2008), as would be

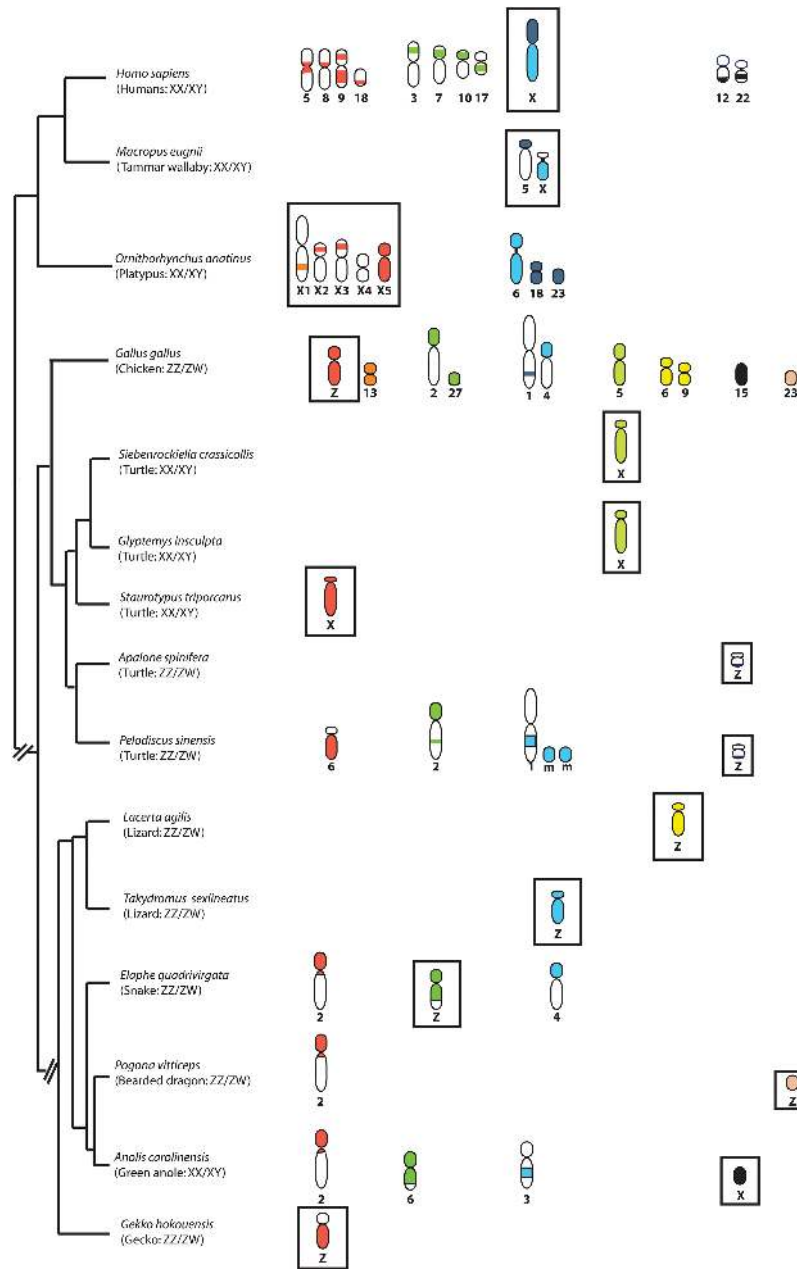


Figure 1. Orthologous segments of sex chromosomes in amniotes. Segments orthologous to the chicken ZW are in red; those orthologous to the snake ZW are in green and segments orthologous to the mammal XY are in blue (light for XCR and dark for XAR), turtles, different lizards, and chicken chromosome 13 are in light green, yellow, black, orange and light orange. For reptiles, we have included all species where sex chromosomal genes were mapped. For mammals and birds, we only included representative species because usually sex chromosomal genes are often highly conserved within a group. The orthologous segments in the amniotes are obtained from the following sources: Grützner et al. (2004); Matsuda et al. (2005); Matsubara et al. (2006, 2012, 2014a); Veyrunes et al. (2008); Kawai et al. (2009); Ezaz et al. (2009, 2013); Kawagoshi et al. (2009, 2012, 2014); Alföldi et al. (2011); Badenhorst et al. (2013); Srikulnath et al. (2014); Rovatsos et al. (2016); Montiel et al. (2016a).

expected if an original bird-like sex chromosome had been sequentially translocated to 4 autosomes. *AMH* is currently the best candidate for the monotreme sex-determining gene (Cortez et al. 2014).

Bird Sex Chromosomes

Like mammals, birds have a very stable sex chromosome system, but it is female, rather than male heterogametic. The avian Z chromosome is highly conserved in size and morphology across all bird families. Comparative chromosome painting and sequence analysis showed high sequence homology across the most distantly related birds, and physical mapping revealed high levels of linkage homology

(Shetty et al. 1999; Shibusawa et al. 2004; Nishida-Umehara et al. 2007; Zhou et al. 2014). Sex in birds appears to be determined by the dosage of *DMRT1* which lies on the Z but not the W (Smith et al. 2009), although cell autonomous factors are also involved (Clinton et al. 2012).

It has long been known that the bird ZW pair shows no homology with the therian XY pair. Instead, the chicken Z chromosome shares homology with human chromosomes 5, 9, and 18, and the human X chromosome shares homology with chicken chromosomes 4 (XCR) and 1 (XAR) (Nanda et al. 1999; Nanda and Schmid 2002; Kohn et al. 2004) (Figure 1).

Reptile Sex Chromosomes

Sex determination and sex chromosomes of non-avian reptiles demonstrate great variety. The ancient tuatara, all crocodylians, most turtles, and some lizards have TSD (reviewed by Ezaz et al. 2006). Many lizards and some turtles exhibit genotypic sex determination (GSD), and both male and female heterogamety (XY, X₁X₂Y, ZW, and Z₁Z₂W) may be found within in the same taxa. Their sex chromosomes show a range of differentiation between homomorphic and heteromorphic sex chromosomes (Ezaz et al. 2009c; Olmo 2013).

Snakes (Serpentes) are unusual among reptiles in their conserved ZZ/ZW-type sex chromosomes. The Z chromosome is similar in size across species, but the W chromosomes vary from homomorphic in birds to highly differentiated in other families. Quantitative PCR and molecular gene-specific DNA markers revealed a high level of Z chromosome conservation in snakes (Rovatsos et al. 2015; Laopichienpong N, et al., unpublished data), and full sequencing confirmed genetic homology (Vicoso et al. 2013). The snake sex-determining gene is unknown.

Although the Z chromosomes of snakes and birds are similar in size, they share no genes. Comparisons of physical maps and whole genome sequence analysis revealed that chicken Z-borne genes lie on the short arm of snake chromosome 2, and chicken homologs of snake Z-linked genes lie on chicken chromosomes 2 and 27 (Matsubara et al. 2006, 2012; Vicoso et al. 2013). However, although they are nonhomologous, bird and snake W chromosomes share blocks of 3 repetitive sequences (O'Meally et al. 2010).

The sex chromosomes of lizards are notoriously varied. The Z chromosome of the Hokou gecko (*Gekko hokouensis*) but not the marble gecko (*Christinus marmoratus*) has homology with the chicken Z chromosome (Kawai et al. 2009; Matsubara et al. 2014a). Rapid evolution of nonhomologous sex chromosomes has been reported within closely related species. For example, within Australian dragon lizards, comparative mapping of a sex chromosome-specific marker showed that the ZW sex microchromosomes of 3 Australian agamid species are homologous, but that those of a fourth species, are not (Ezaz et al. 2009b). This suggests that sex chromosome turnover occurred among closely related species within each subfamily as well as between the 2 families of lizards (gecko and dragon).

The draft genome assemblies of the green anole lizard (*Anolis carolinensis*) and chicken facilitate detailed comparisons of their sex chromosomes (Alföldi et al. 2011) and provide new perspectives on the comparative genomics of amniote sex chromosomes. The green anole has an X microchromosome whose linkage group is conserved through the family Iguanidae (Gamble et al. 2014; Rovatsos et al. 2014). This X is homologous to chicken chromosome 15 (Alföldi et al. 2011). However, outside this clade, the micro-Z chromosome of the bearded dragon lizard (*Pogona vitticeps*) and the Z chromosome of the sand lizard (*Lacerta agilis*) have homology with chicken chromosomes 23, and 6 and 9, respectively (Ezaz et al. 2013; Srikulnath et al. 2014) (Figure 1). Interestingly, the Z chromosome of the 6-striped long-tailed lizard (*Takydromus sexlineatus*), a species closely related to the sand lizard, is homologous to the chicken chromosome 4p, a region that corresponds to the mammalian X chromosome (Rovatsos et al. 2016).

Orthology to the chicken Z chromosome was also found in the Mexican giant musk turtle (*Staurotypus triporcatus*) and the giant musk turtle (*Staurotypus salvinii*) (Kawagoshi et al. 2014). However, the X chromosome of the marsh turtle (*Siebenrockiella crassicollis*) and the wood turtle (*Glyptemys insculpta*), and the Z chromosome of the Chinese soft (*Pelodiscus sinensis*) and the spiny softshell turtle

(*Apalone spinifera*) correspond to chicken chromosomes 5 and 15, respectively (Kawagoshi et al. 2009, 2012; Badenhorst et al. 2013; Montiel et al. 2016b) (Figure 1).

Sex Chromosome Turnover in Amniotes

The variety of nonhomologous sex chromosomes in amniotes is understandable in terms of the mechanisms by which novel sex chromosomes arise. Theory predicts, and many observations confirm, that novel sex chromosomes are defined by the acquisition of novel sex-determining genes. We now have several examples of how new sex-determining genes have arisen in vertebrates.

The best examples of evolution of de novo sex-determining genes come from fish and frogs rather than amniotes. Medaka fish (*Oryzias latipes*) and their relatives have similar karyotypes, but different chromosomes are sex determining. In medaka, acquisition of a copy of *DMRT1* defined a neo-Y, whereas in other species, the growth factor *GSDf*, or its upstream regulator *SOX3*, became sex determining (Kikuchi and Hamaguchi 2013). Similarly, a truncated copy of *DMRT1* defines a new W chromosome in the clawed frog (*Xenopus laevis*) but not in the western clawed frog (*X. tropicalis*), and in the Japanese frog, *Grandirana rugosa*, *SOX3* has become sex determining (Uno et al. 2008; Yoshimoto et al. 2008; Roco et al. 2015).

Sex Chromosome Turnover in Reptiles

Without information on reptile sex-determining genes, we are unable to detect such turnovers of sex genes directly in reptiles.

However, it is possible to detect turnover at the chromosome level in the numerous examples of sex chromosomes that are autosomal in related lineages. For instance, comparative gene mapping shows that the sex chromosomes of the dragon lizard *P. vitticeps* are not homologous with those of snakes or chicken (Ezaz et al. 2009a).

Sex chromosome turnover in reptiles is facilitated by numerous changes from TSD to GSD and back again. For instance, in the phylogeny of Australian agamid lizards spanning only 15–24 million years, TSD and GSD have evolved at least 6 times and in at least one instance involved nonhomologous ZW sex chromosomes (Ezaz et al. 2009b).

The discovery that several reptiles combine genetic with temperature sex determination (Quinn et al. 2007) suggested a mechanism by which changes from GSD to TSD could readily occur, simply by raising the threshold of a dosage-regulated sex-determining product to produce ZZ females as well as ZZ males (Bull 1985; Quinn et al. 2011). Lowering the threshold would produce ZW males as well as ZW females, resulting in a transition to a WW female: ZW male system, which is recognizable as an XY system.

Recent work has demonstrated the ease with which a GSD species with a temperature override can move from GSD to TSD in a single generation (Holleley et al. 2015). This species has a ZZ male:ZW female system, but when eggs are incubated at a high temperature, ZZ as well as ZW embryos develop as females. The ZZ sex-reversed females are viable and fertile. When mated with ZZ males, they produce all ZZ offspring whose sex is entirely dependent on incubation temperature.

Sex Chromosome Turnover in Mammals

Although we cannot be sure of the original sex-determining system in the ancestral mammal, the homology of the oldest platypus XY pair to the chicken ZW suggests a bird-like ZW system that was

either ancestral or convergently evolved. This underwent radical turnover both in the monotreme and the therian lineages. In monotremes translocation of an *AMH*-bearing autosome provided an alternative sex-determining locus and set in train a cascade of serial translocations that resulted in the bizarre multiple XY system (Rens et al. 2007).

In therians, the evolution of *SRY* from *SOX3* defined a novel pair of sex chromosomes that became the therian XY (Graves 2013). *SOX3* is a highly conserved gene in vertebrates. In mammals, it is normally expressed in the central nervous system and in germ cells. However, ectopic expression in the somatic cells of the gonad in transgenic mice, and in human patients, produces XX male sex reversal (Sutton et al. 2011), suggesting that all that was required to acquire a sex-determining function was a mutation or insertion of a promoter that drove expression into the developing gonad.

Mammals also provide us with some very recent examples of turnover of the sex genes, and sex chromosomes. In 2 rodent lineages (2 out of 3 species of the mole vole *Ellobius*, as well as 2 of 3 species of the spiny rat *Tokudaia*), the degenerate Y chromosome, and *SRY* have been completely lost (Just et al. 1995; Kuroiwa et al. 2011; Kimura et al. 2014). Fertility genes have been moved, copied, or replaced. There is now evidence that in the spiny rat, an amplified *CBX2* gene on chromosome 1 has taken over the role of initiating the sex determination pathway (Kuroiwa et al. 2011; Murata et al. 2012; Kimura et al. 2014). This gene, with *SF1*, has an upstream role in the conserved vertebrate sex determination pathway but may have taken over a master switch role in the Y-less spiny rats (Kimura et al. 2014).

How Did Ancestral Amniotes Determine Sex?

The great variety of systems in mammals, birds, and reptiles, and the rapidity of their turnover in reptiles, makes it difficult to deduce what system was used in a common ancestor 310 MYA. Ancestral reptiles could have had an environmental sex-determining mechanism (as do many reptiles in several different reptile orders), or any one of several genetic systems of male or female heterogamety, with more or less differentiated sex chromosomes.

Was it an environmental system that independently spawned different systems of genetic sex determination? Or a GSD system that diversified as old systems died and new ones were reborn, and independently slipped into an environmental mode in several lineages? An in-depth phylogenetic reconstruction is needed because of the wide distribution of both TSD and GSD systems among different reptilian orders, and the frequent flip-flops between them.

Pokorná and Kratochvíl (2016) have proposed, from phylogenetic context, that the ancestral reptiles had TSD. They argue that TSD to GSD transitions are more likely than GSD to TSD because “an individual with a mismatch between phenotypic (gonadal) and genotypic sex, for example an individual sex reversed by environmental effects, should have a lower fitness due to the lack of specialized, sex-specific parts of their genome.” However, there is little observational evidence for the generality of this statement, and we recently showed that ZZ female dragon lizards sex reversed by high-temperature incubation lay more eggs which have a better hatching success than their normal ZW sisters (Holleley et al. 2015). They are fitter in the wild too, since the proportion of ZZ females has increased over the past 10 years.

The question therefore remains open. The report of homology between amniote and salamander sex chromosomes, and the recent observation that some turtle sex chromosomes have homology to amphibian sex chromosomes, may imply that some sex chromosome functions predated reptile evolution (Montiel et al. 2016a, 2016b).

Decommissioning Sex Chromosomes

After sex chromosome turnover, does the old sex chromosome pair revert to an autosomal state? There is gathering evidence that its history as a sex chromosome stamps it indelibly as an ex-sex pair.

For instance, the XY pair of *Drosophila melanogaster* is not shared by other dipteran families. Rather, many of these flies have a small dot-like chromosome as the sex pair (Vicoso and Bachtrog 2013, 2015). The genetically homologous dot-like chromosome 4 of *D. melanogaster* has long been a mystery because it is small, almost devoid of genes, and does not recombine, all trademarks of sex chromosomes. This suggests that its history as a sex chromosome pair has permanently altered its gene content and behavior at meiosis.

Sex chromosome turnover has also occurred in the last few million years in Y chromosome-less rodents, so it is possible to examine X chromosomes that have been recently decommissioned. In 2 species of spiny rat that have lost the Y chromosome and *SRY* and evolved a new system of sex determination by amplification of *CBX2* on a neo-Y, the original X has not reverted to an autosomal state, for it is present in only a single copy in both sexes (Kuroiwa et al. 2011). This may be because genes on the single X in males were overexpressed as a dosage compensation mechanism in an XY ancestor as well as XO species, and a compensatory X inactivation mechanism in females was lost, so that 2 X chromosomes would be lethal.

The same situation is found independently in the mole vole *Ellobius lutescens*, which has lost the Y chromosome and *SRY* (Just et al. 1995) in favor of an unknown novel sex gene, and in which both sexes are XO. Related species *E. tancrei* and *E. talpinus* have XX males and females, fulfilling the expectations that this chromosome has reverted to an autosomal state. However, this is evidently not the case, because at male (but not female) meiosis, the 2 X chromosomes form a heterochromatic sex body and fail to recombine along most of their length (Fredga 1983; Marveevsky et al. 2016).

Hints of Ancient Orthology in Amniotes: Was There a Super-Sex Chromosome?

Our knowledge of how novel sex genes evolve leads to the expectation that almost any autosome can become a sex chromosome. However, this expectation is not borne out. The same orthologous genes have been discovered to have a sex-determining function in several different lineages (O’Meally et al. 2012). This has led to the convergent evolution of the same region as a sex chromosome pair in widely different amniote lineages.

However, this does not explain the curious finding that quite different sex chromosomes that are defined by different sex-determining genes share synteny in other vertebrates. A spectacular example, in which the mammal XCR, XAR, and chicken Z chromosomes all lay together on a large autosome in the salamander *Ambystoma*, was detailed by Smith and Voss (2007) and suggested to result from a common origin (Smith and Voss 2007). However, it remained possible that such a huge chromosome had a high probability of containing unrelated segments just by chance (O’Meally et al. 2012).

Comparative gene mapping studies now identify several other striking homologies between the sex chromosomes in very distantly related amniotes (summarized in Figure 1). We detail here some overlaps of orthologous segments among amniotes and speculate on their interpretation.

As shown previously, the nonhomologous W chromosomes of birds and snakes share at least 3 families of amplified repetitive sequences (O’Meally et al. 2012) which are not present elsewhere

in the genomes analyzed (O'Meally et al. 2010). This suggests that they might have been part of a larger sex chromosome in a common reptilian ancestor. In addition to this relationship, we find that monotreme sex chromosomes contain most of the chicken Z chromosome (largely as X_3 , and segments of X_2 and X_3 ; X_1 with homology to chicken chromosome 13 [Veyrunes et al. 2008; Matsubara et al. 2012] that corresponded partially to squamate chromosome 2). However, segments from 4 platypus X chromosomes (X_1 , X_2 , X_3 , and X_5) share segmental orthologies with squamate chromosomes 2 (Figures 2 and 3). These intriguing orthologies of several genes between multiple XY chromosomes of platypus and chicken ZW chromosomes suggest that there may have been a conserved ancestral amniote chromosome, fission of which gave rise to reptilian, monotreme, and therian sex chromosomes (Rens et al. 2004; El-Mogharbel et al. 2007; Rens et al. 2007; Veyrunes et al. 2008; Uno et al. 2012; Montiel et al. 2016a).

Importantly, comparative genomics, based on chromosome painting and gene mapping for several squamate reptiles, revealed

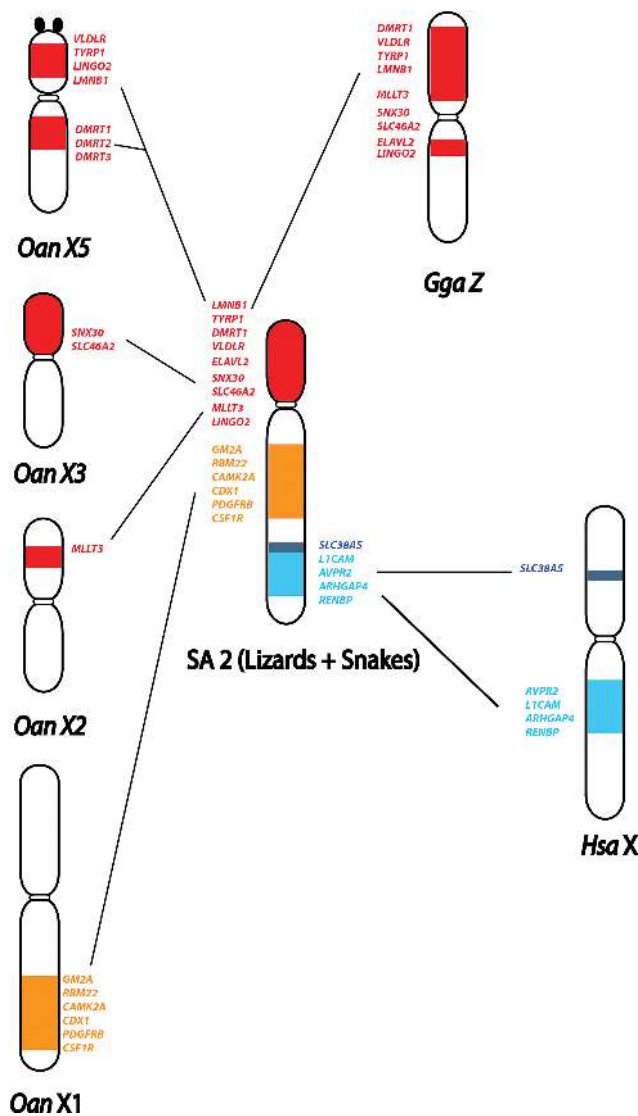


Figure 2. Ancient segmental orthology of amniote sex chromosomes with squamate chromosome 2. *Gga*: *Gallus gallus* (chicken), *Hsa*: *Homo sapiens* (humans), *Oan*: *Ornithorhynchus anatinus* (platypus), SA 2 (squamate autosome 2).

that the chicken Z chromosome was homologous to the short arm of the bi-armed macrochromosome pair in most squamate reptiles, except for lacertid lizards and geckos (Srikulnath et al. 2009, 2013, 2014, 2015; Pokorná et al. 2011). The homologies of chicken sex chromosomes (=platypus sex chromosomes) to chromosome 2 in squamate reptiles led us to investigate in silico orthologies of candidate genes from *A. carolinensis* chromosome 2 (=squamate chromosome 2) with chicken and platypus sex chromosomes. We found that several genes from *A. carolinensis* 2 (=squamate chromosome 2) have retained orthology to platypus X chromosomes (X_1 , X_2 , X_3 , and X_5) as well as to the avian Z chromosome. In addition, *A. carolinensis* chromosome 2 also shares some orthologous genes with the human X chromosomes (Figures 2 and 3), while the human chromosome X shares homologies with small regions of anole chromosome 1, 2, and 3 (Louis et al. 2014). Is this homology coincidental or does it represent an ancient ancestral amniote super-sex chromosomes?

Discussion

Our observation that sex chromosomes in different amniote species share synteny in several amniote species might suggest that different amniote sex-determining regions were part of an ancestral super-sex chromosome. This is an extension of the hypothesis put forward by Smith and Voss (2007) to explain the occurrence of mammal XY and bird ZW genes on a single salamander autosome.

Such a relationship is difficult to comprehend on our present understanding of how new sex chromosomes arise. Here, we discuss some possibilities for alternative explanations.

Several of these alternatives relate to characteristics common to sex chromosomes that are unrelated and defined by different specific sex-determining genes. Not only does the sex-specific chromosome (Y or W) suffer from reduction of active genes and accumulation of repetitive sequences, but its partner (X or Z) undergoes changes in gene content as a result of sex-specific selection in the hemizygous state. In addition, differentiated sex chromosomes share the problem of dosage differences in male and female, which has led to the selection of several different mechanisms for dosage compensation (Graves 2016).

Does Co-location Simply Reflect Chance Rearrangement?

Perhaps the shared synteny of amniote sex chromosomes is nothing more than chance within a restricted set of species. The first example of segmental orthology was the co-location in the salamander *Ambystoma* of the conserved and added regions of mammal sex chromosomes, as well as the bird ZW (Smith and Voss 2007). However, the autosome that harbored them is very large, and the coincidence could have arisen by chance (O'Meally et al. 2012).

It remains possible that the segmental orthologies of squamate chromosome 2 to platypus, avian, and human sex chromosomes and to dragon (*P. vitticeps*) sex microchromosomes that we detected (Srikulnath et al. 2009, 2013, 2015; Ezaz et al. 2013; Young et al. 2013; Ezaz T, unpublished data) represents random homologies, as they are based on only small sets of genes in a restricted set of species (Figure 1). However, comparisons outside amniotes strengthen the evidence for identity by descent. For instance, *X. tropicalis* chromosome 1 contains genomic blocks that correspond to chicken sex chromosomes, several turtle sex chromosomes, and 2 lizard species sex chromosomes (*G. hokouensis* and *A. carolinensis*). This suggests that *X. tropicalis* chromosome 1 represents a proto-sex chromosome

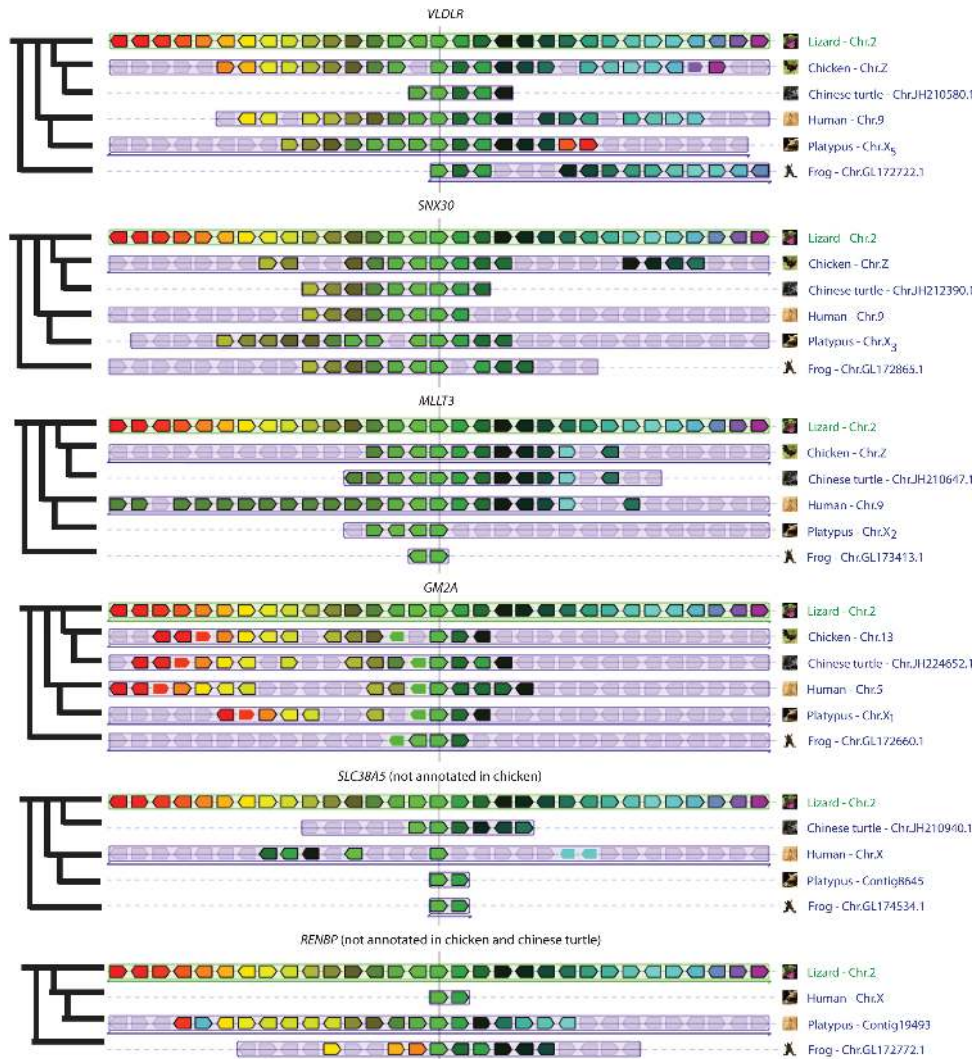


Figure 3. Segmental orthology of squamate (*Anolis carolinensis*) chromosome 2 genes with those of chicken (*Gallus gallus*), Chinese turtle (*Pelodiscus sinensis*), platypus (*Ornithorhynchus anatinus*), human (*Homo sapiens*), and frog (*Xenopus laevis*). Chinese turtle and frog are included as outgroup of squamates-birds and amniotes, respectively. The segmental synteny and animal pictures are derived from the Genomic database (Louis et al. 2014). Anchored genes are those from Figure 2. The same color and orientation indicate orthologous genes.

for other amniote sex chromosomes (Uno et al. 2012; Brelsford et al. 2013; Montiel et al. 2016a, 2016b).

Does Co-location Reflect Co-option of Particularly Favorable Genes or Chromosomes?

Several sex-determining genes are now known in several different vertebrates, and it is striking that orthologous or paralogous of the same few genes turn up repeatedly in distantly related animals. For instance, the bird sex-determining gene *DMRT1* is also sex determining in the tongue sole, and copies of it determine sex in Medaka fish and *X. laevis*, as described above. *SOX3*, the ancestor of the human *SRY*, is sex determining in another *Oryzias* species, as well as the Japanese wrinkled frog, and *AMH* or its receptor is sex determining in distantly related fish species, as well as monotreme mammals (Cortez et al. 2014). Since they cannot all be identical by descent, we conclude that these genes are particularly suited to controlling the sex determination pathway, and they, or copies of them, have independently acquired a sex-determining function in many lineages.

This could certainly explain why a particular synteny group is associated with sex in distantly related lineages, since genes that are

physically linked to one of these repeatedly chosen sex-determining genes will hitchhike with it, especially since sex chromosomes tend to be rearranged infrequently. However, this does not explain why 2 synteny groups, hitchhiking to form sex chromosomes in different lineages, would be found together in an ancestor.

Another possibility is that a second potentially sex-determining gene has a advantage in the same sex which is designated by the sex-determining gene. At least some of the sex-determining genes, such as *DMRT1* and *AMH*, are involved in the sex determination pathway even where they are not the head of it. It is possible, therefore, that there has been selection for a male (or female-) specific allele on the chromosome that carries the gene that specifies that sex. These genes could have acted as the sex-specific gene that drove selection for the loss of recombination with the sex-specific member of the sex pair (Rice 1984; Charlesworth 1991).

Does Co-location Reflect Interphase Chromosome Position?

Another hypothesis is that these chromosomes happen to occupy adjacent positions in the interphase nucleus in all amniotes. This

could make their physical fusion and translocation more likely, as seems to be the case for translocations in cancer (Othman et al. 2012).

Chromosome painting shows that chromosomes occupy highly conserved territories both in somatic cells of mammals and birds (Tanabe et al. 2002; Gilbert et al. 2005; Skinner et al. 2009) and mammalian germ cells (Greaves et al. 2003). The positions of these territories are related to the gene content of chromosomes, and sex chromosomes, having a low gene density, are more frequently at the periphery. The paucity of genes on sex chromosomes appears to remain a property of ex-sex chromosomes, as we have discussed.

It is also apparent that the interphase position of chromosomes is related to their epigenetic state, which may have relevance to dosage compensation of sex chromosomes, at least in therian mammals, although evidently not for snakes and birds (McQueen et al. 2001; T. Cremer and M. Cremer 2010; Wolf and Bryk 2011; Vicoso et al. 2013). The original bird-like sex chromosome of a mammal ancestor may have undergone serial translocations with the chromosomes in closest proximity, forming X_1 , X_2 , and X_3 in a monotreme ancestor. It is interesting that platypus chromosome 6, the homologue of the mammalian XCR, lies adjacent to the sex chromosome chain at meiosis (Cortez et al. 2014); perhaps it will be next to undergo translocation into the sex chromosome chain. Perhaps this relationship was also important for the choice of this autosome as the therian sex chromosome 190 MYA.

Does Shared Synteny Reflect Interactions of Sex-Determining Genes?

We know that there are several genes that are sex determining in one species, but not in others. The expectation is that these genes have been independently recruited to head the sex-determining pathway in different species, where each is necessary and sufficient for sex determination. Is there an alternative whereby 2 sex-determining genes must act in concert?

Most genes that are sex determining in one species are part of the conserved sex-determining network in all lineages. For instance, *DMRT1*, although it does not initiate sex determination in mammals or snakes, is essential for testis stabilization, and *AMH*, although it is not sex determining in therian mammals or birds, is essential for male development. Other sex-determining genes arose from transcription factors or growth factors that were recruited to a novel sex-determining role. Is it possible that the biochemical interaction of the products of these genes to bring about sex determination is facilitated by their physical proximity?

There is no evidence that this is so. Indeed, transgenesis and knockouts imply the exact opposite; that *SRY*, by itself is male determining in mammals (Koopman et al. 1991), and *DMRT1*, by itself, exerts dose-dependent control of sex determination in birds (Smith et al. 2009).

Are There Interactions Between Other Genes or Sequences on Sex Chromosomes?

There are many changes that occur to gene content once an autosome pair becomes a sex chromosome pair. Not only is there mass deletion, and selection of sex-specific traits on the sex-specific chromosome (Y or W), but there are also changes in the gene content of the partner sex chromosome (X or Z) that reflect its hemizygous state in one sex. For instance, the mammal X has accumulated and amplified testis antigen genes and other genes involved in reproduction as well as intelligence. The chicken Z has also accumulated genes involved in female reproduction (Mank 2013). As we have

discussed, this altered gene content remains a property of ex-sex chromosomes for millions of years.

It is hard to see how such changes in gene content of an ancestral sex chromosome predispose regions of it to become sex chromosomes in different lineages. However, particular sequences such as ribosomal RNA genes may play some part in sex chromosome regulation. Several mammalian sex chromosome pairs (e.g. opossum and kangaroo) have independently fused with a segment carrying the nucleolus organizing region (NOR). It has been observed that platypus sex chromosomes are frequently arrayed around the nucleolus during meiosis, which brings them into close proximity to the NOR-bearing chromosome 6, the homologue of the human XCR. Proximity to the site of RNA synthesis might facilitate epigenetic processes involving long noncoding RNAs.

The NOR may also be important in rearrangements that create novel sex chromosomes. The NOR is generally located on a pair of microchromosomes or chromosome 2 in Iguania (Porter et al. 1991). Our 2 color fluorescence in situ hybridization mapping of the NOR along with bacterial artificial chromosome (BAC) clones derived from ZW sex microchromosomes in bearded dragon lizard revealed that the sex chromosome-derived BAC clone contains repeats shared with the region adjacent to NOR repeat location in chromosome 2. This led us to hypothesize that the sex microchromosomes in this species may have arisen by fission adjacent to NOR near the telomere of chromosome 2 and later fusion with a proto microchromosome. These rearrangements provide evidence that the bearded dragon ZW pair once shared a common ancestry with squamate chromosome 2 (Ezaz et al. 2009a, 2013; Young et al. 2013).

Another possibility is that the behavior of sex chromosomes may relate to their content of repetitive sequence. Human and mouse X chromosomes have been enriched with long interspersed nuclear elements (LINEs) that are thought to act in transmitting epigenetic silencing on the inactive X (Lyon 1986). The epigenetic marker CCCTC-binding factor (CTCF) also appears to act as insulators of spreading of inactivation along the inactive mouse and human X chromosome (Disteche 2012). CTCF sequences are highly conserved epigenetic regulators (Hore et al. 2008) that link promoters and enhancers and correlate with gene activity.

Thus, the puzzling observation that the genetically nonhomologous W chromosome of birds and snakes share several repetitive sequences (O'Meally et al. 2012) might suggest that these repetitive sequences have a function in chromosome conformation that make these regions more likely to become successful sex chromosomes.

Does Shared Synteny Reflect Conformation of Sex Chromosomes?

Rather than look for an explanation for shared synteny in novel sex-determining genes, or in the altered gene or repeat content of sex chromosomes, perhaps we must look more generally at the properties of sex chromosomes as a whole. Are there changes in properties of chromosomes, for instance reduction of recombination, altered 3D conformation and dosage compensation that make parts of them more likely to become sex-determining regions in descendants?

Recent examination of the 3D architecture of human chromosomes using in situ Hi-C (Rao et al. 2014) shows that genomes are partitioned into short (185kb) contact domains that contain loops anchored at CTCF sequences. Such 3D interactions appear to be critical for gene expression along chromosomes (Dekker and Mirny 2016). Although the active X chromosome has a structure similar to that of the autosomes, the inactive X chromosome has a bipartite structure in both human and mouse; 2 massive domains that are

separated by a similar hinge sequence (Rao et al. 2014; Deng et al. 2015). Thus, the 3D structure of the inactive X results from long-range interactions between sequences of the inactive X chromosome.

It may be, therefore, that evolution of control sequences such as LINEs and CTCF on sex chromosomes that must be dosage compensated, remain on decommissioned sex chromosomes and predispose regions to become sex chromosomes in other lineages.

Conclusions

The diversity of sex chromosomes among amniotes can be understood as the product of independent evolution of different systems in different lineages, defined by novel sex-determining genes. It is also easy to understand the numerous instances of convergent evolution of sex chromosomes across distantly related taxa if certain genes are particularly adept at taking on a sex-determining role.

However, it is more challenging to explain why nonhomologous regions that are sex determining in one lineage or another are found to share synteny in several different lineages. Although the first example of shared synteny (mammal X and bird Z lay on the same chromosome in a salamander (Smith and Voss 2007) could be dismissed as sheer coincidence, comparative gene mapping and more recently whole genome sequencing, have now turned up other surprising relationships among mammals, birds, turtles, snakes, and lizards.

Does this, after all, constitute evidence that these regions were once linked in a super-sex chromosome that was broken up in different ways in different amniote lineages? Or does it signify that special properties of sex chromosomes (paucity of active genes, low recombination, epigenetic regulation to achieve dosage compensation) predispose particular chromosomes to a sex-determining role?

The advances in genomics technologies, particularly low-cost next generation sequencing, provide unparalleled opportunities for in-depth analysis of sex chromosomes—and recently decommissioned ex-sex chromosomes—in other amniotes that have recently undergone sex chromosome turnover. It will be valuable to compare maps and sequences and explore homologies across wider evolutionary distances. It will also be crucial to examine sequences involved in conformation, recombination, and epigenetic regulation of gene dosage of sex chromosomes, and of ex-sex chromosomes. Combinations of short- and long-read sequencing can also provide chromosome-scale descriptions of repeat landscape of sex chromosomes, providing insights into the origin and degeneration of sex chromosomes, as well evidence of conservation of repetitive sequences on sex chromosomes across taxa. With ever more genomes being sequenced, these technologies can also provide opportunities to explore and investigate the epigenetic landscape of young and cryptic sex chromosomes, to determine their evolutionary origin as well as explore their stability and functions. These new sequence information will then provide basis for a high resolution phylogenetic reconstruction, which will combine existing databases such as Ensembl (Flicek et al. 2014) and Genomicus (Louis et al. 2014), and enable dense comparative physical mapping. This will indeed be required to test the alternative hypotheses raised in this paper.

There is particular value in examining animals—reptiles, amphibians, and fish as well as mammals—with atypical sex chromosomes and sex determination, and it is heartening to see the rapid accumulation of data from sex chromosomes from whole groups such as the birds (Zhou et al. 2014) that has resulted from the Genome 10K project (Koepfli et al. 2015).

Funding

This study was partially supported by an Australian Research Council Future Fellowship (FT110100733, awarded to T.E.) and Professor Motivation (PM) and Science Research Fund (ScRF) from the Faculty of Science and the Fellowship of Capacity Building for Kasetsart University on Internationalization, Kasetsart University, Thailand awarded to K.S.

Conflict of Interest

The authors declare no conflict of interests.

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