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# Review

# Sex determination in mammals – Before and after the evolution of *SRY*

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**Abstract.** Therian mammals (marsupials and placentals) have an XX female:XY male sex chromosome system, which is homologous to autosomes in other vertebrates. The testis-determining gene, *SRY*, is conserved on the Y throughout therians, but is absent in other vertebrates, suggesting that the mammal system evolved about 310 million years ago (MYA). However, recent work on the basal monotreme mammals has completely changed our conception of how and when this change occurred. Platypus and echidna lack *SRY*, and the therian X and Y are represented by autosomes, implying that *SRY* evolved in therians after their divergence from monotremes only 166 MYA. Clues to the ancestral mechanism usurped by *SRY* in therians are provided by the monotremes, whose sex chromosomes are homologous to the ZW of birds. This suggests that the therian X and Y, and the *SRY* gene, evolved from an ancient bird-like sex chromosome system which predates the divergence of mammals and reptiles 310 MYA.

**Keywords.** Mammalian sex determination, evolution, *SRY.*

## Introduction

Sex is vital for the reproduction of all vertebrates, so it is surprising that the mechanisms by which maleness and femaleness is determined are so variable between taxa. Many different sex-determining systems have evolved in vertebrates. These mechanisms can be broadly categorized as either environmental sex determination or genetic sex determination. Environmental sex determination is widely employed in fish, where a range of stimuli from social cues to temperature establishes sex. Temperature sex determination is also extensively utilized in reptiles.

Some fish and reptiles, and all amphibians, birds and mammals have genetic sex-determining systems. In some species, genetic sex determination is easy to identify by the presence of heteromorphic sex chromosome pairs that bear the sex-determining gene. In other species, the sex chromosomes are homomorphic and unable to be distinguished microscopically (cryptic). Some sex chromosomes are tiny (*e.g.*, the micro sex chromosomes of some turtles) and others large (*e.g.*, in mammals, and the macrochromosomes of birds and snakes). Sex chromosomes may be highly conserved, as in mammals and birds, or variable even within species, as for some frogs. The sex-determining gene, and its mode of action, sometimes differs completely between even closely related species.

We know most about the sex chromosomes of humans, which have a male heterogametic XY male:XX female system in which maleness is determined by a dominant gene on the Y (*SRY*) that triggers a cascade of events leading to testis development. The X and Y

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are heteromorphic: the X is large and euchromatic and highly conserved, whereas the Y is small, gene poor, repetitive and half heterochromatic. The gene content of the Y varies between species, but *SRY* orthologues have been identified on the Y in both branches of therian mammals, placentals (infraclass Eutheria) and marsupials (infraclass Metatheria), implying that this sex-determining mechanism was present before their divergence 148 million years ago (MYA) (reviewed in [1]). All groups of therian mammals possess an *SRY* gene on the Y chromosome except for a few exceptional rodents such as the mole voles [2] and Japanese country rats [3, 4], which have lost their Y (and *SRY*) recently.

Some other vertebrate species, such as birds and snakes, have a ZZ male:ZW female sex chromosome system. Gene mapping experiments show that the therian XY pair has no homology to the bird ZW [5], and the snake ZW pair is different again [6]. The therian sex chromosomes are homologous to autosomes in non-mammalian vertebrates, and the bird and snake ZW pairs are homologous to autosomes in therian mammals [5–7]. The finding that sex chromosomes in one lineage may be homologous to autosomes in other lineages is consistent with the hypothesis that sex chromosomes evolved independently from autosomes that have acquired a sex-determining allele [8]. New sex-determining alleles are proposed to "hijack" a conserved vertebrate sex-determining pathway [7], and may be selected for if conditions favor a sex bias, or in response to a sex imbalance [9].

A considerable shock to our understanding of mammal sex chromosomes and sex determination was dealt by our recent discovery that the basal monotreme mammals (subclass Prototheria), which diverged from Theria 166 MYA, do not share the same XY system as therians, and lack an *SRY* orthologue [10–13]. The therian X and Y are homologous to autosomes in monotremes, as in non-mammalian vertebrates, which also lack *SRY*. *SRY* is therefore unique to the therian mammals, and the evolution of *SRY*, as a new sex-determining switch, presumably initiated the differentiation of the therian X and Y sex chromosome pair 166–148 MYA (Fig. 1). Sex-specific selection has subsequently endowed the X with an over-representation of sex and intelligence genes, while the Y has been degraded and heterochromatinized (reviewed in [14]). The Y retains only a small fraction of its original gene content, and uniquely in the genome, is specialized for reproductive function.

A bombshell was the finding that genes on monotreme sex chromosomes map to the bird ZW pair [11, 13, 15, 16]. Monotremes (platypus and echidna) most recently shared a common ancestor with birds, which cluster within reptiles, 310 MYA. The homology of the avian and monotreme sex chromosomes raises the possibility that these systems had a common origin in the ancestor of amniotes.

In light of this startling new information, here we review for the first time data from which we can deduce the sex-determining state of the ancestral mammal, which may have utilized extremely ancient sex chromosomes predating the divergence of reptiles and mammals. This will help us to gain an understanding of how *SRY* evolved in the first therian mammals, and of the pathway of which *SRY* only recently assumed control.

## Sex chromosomes of therian mammals

Therian mammals have two heteromorphic sex chromosomes (X and Y) that are differently represented in males and females. In humans, the sex chromosomes are very different in size and gene content; the X is a mid-sized chromosome bearing about 1300 genes in 155 Mb [17], whereas the Y is roughly a third that size and highly heterochromatic, containing only 45 active genes coding for unique proteins. The male-specific region of the Y chromosome (MSY) contains 156 transcribed units (many of which are pseudogenes or amplified copies) and only 27 unique protein-coding genes [14, 18].

Although not obvious from morphology, gene mapping reveals that the human Y is a highly differentiated copy of the X. The XY pair still share small terminal regions, known as pseudoautosomal regions (PARs), that are homologous and continue to recombine at meiosis. In addition, most genes on the Y chromosome have paralogous copies on the X, from which they obviously diverged. Comparative mapping shows most of the ancient therian X is homologous to the short arm of chicken chromosome 4 [19]. A small separate region of the ancient X with homology to chicken chromosome 12, also separate in fish, was identified from examination of the database [20], but it is not clear whether this is truly orthologous. These data support the hypothesis that the ancient therian X and Y evolved from a homologous autosomal pair that remain as autosomes in other vertebrate lineages.

The differentiation of the therian sex chromosomes from this ancient autosome began when one member of the ancestral autosome pair acquired a new sex-determining allele, defining a proto-Y and proto-X [21]. Other sex-specific alleles accumulated near this locus, and suppression of recombination between the nascent sex chromosomes ensured that the male-specific genes stayed together. In the absence of recombination, the Y chromosome progressively

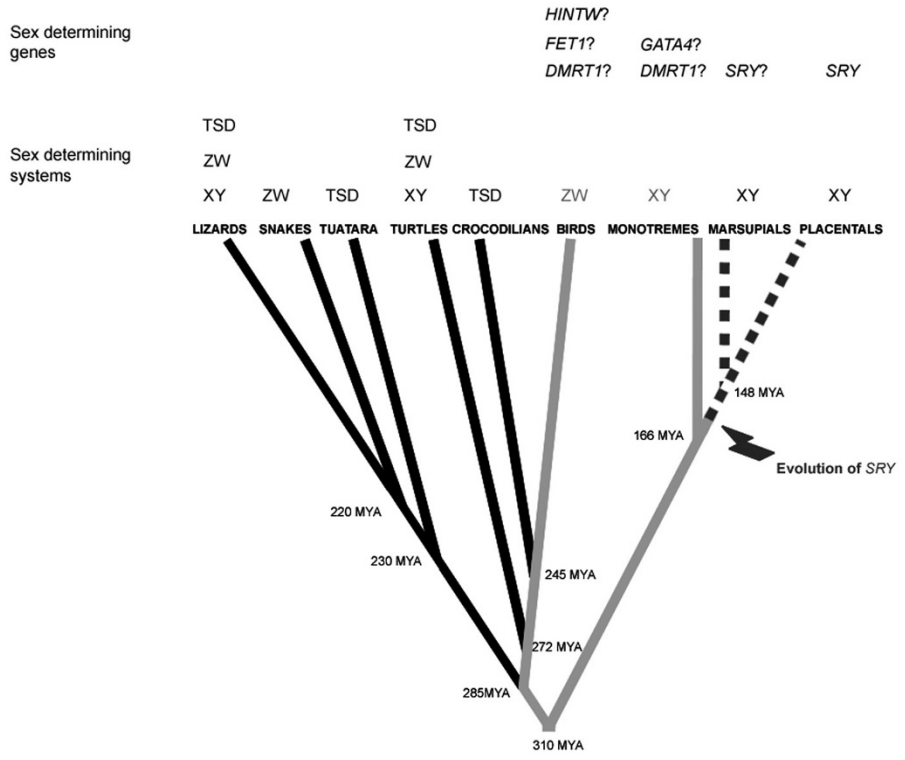


**Figure 1.** Sex-determining systems of amniotes. The homology of the bird and monotreme sex chromosomes infers an ancestral amniote sex chromosome system (gray lines), superseded by *SRY* in therian mammals (dashed lines). Dates of divergence taken from [113, 114]. ESD: Environmental sex determination, TSD: temperature sex determination.

degraded, resulting in the differentiated X and Y chromosomes observed today.

Two main processes have been implicated with the attrition of the therian Y: inefficient selection on a non-recombining chromosome, and a higher mutation rate in the testis (reviewed in [22]). Genes in the non-recombining region of the Y chromosome are rapidly inactivated and lost as point mutations, insertions, deletions and repeated sequences are accumulated by drift (accidental loss of the least mutated Y in a process known as Muller's ratchet). In the absence of recombination, selection is inefficient; a favorable allele can promote an otherwise poor Y (hitchhiking), or be lost due to being on a poor Y (background selection). The higher mutation rate of the Y, compared to the X and the autosomes, is due to its continual passage through the testis, which is a highly mutagenic oxidative environment where germ cells undergo many rounds of replication in the absence of repair enzymes in sperm [23].

Comparisons between the Y chromosomes of placental species reveals that this process has occurred in parallel, as different, although overlapping, subsets of genes have survived on each Y (reviewed in [14]). Humans and mice retain different subsets of genes on the Y, as does the tammar wallaby. A similar process of sex chromosome differentiation has occurred independently in snakes and birds, resulting in the gradual decay of the W. In both these taxa, different families display different extents of W degradation; indeed, the differing degrees of W degradation in snake families was the inspiration for Ohno's hypothesis that vertebrate sex chromosomes differentiated from autosomes [21].

Most of the genes that remain on the therian Y have sex and reproductive functions, presumably having staved off degradation by positive selection. Y genes are highly differentiated and specialized copies of the widely expressed X homologues from which they evolved. For instance, the testis-specific spermatogenesis gene *RBMY* diverged from *RBMX*, which is ubiquitously expressed and may have functions in brain development [24, 25], and *TSPY* (the putative gonadoblastoma gene) diverged from the cell cycle gene *TSPX* [26]. In mice, the X-borne *Sox3* gene, from which *Sry* evolved, is widely expressed in the gonad and central nervous system, whereas *Sry* expression has a much more limited profile in the testis and brain [27].

In contrast to the Y, which has lost nearly all its genes, the X has retained its gene content and is almost identical in all placental mammals. Comparative mapping in marsupials, however, reveals two evolutionary layers [28]. Genes on the long arm and pericentric region of the human X lie on the marsupial X, whereas genes distal to Xp11.23 are autosomal in

marsupials. This defines an ancient X shared by all therian mammals, the X conserved region (XCR), and a region added to the X early in the placental lineage, the X added region (XAR). The XCR corresponds to the evolutionary blocks on chicken chromosome 4p and chromosome 12, and the XAR to a block on chicken chromosome 1. Measuring the sequence divergence between X and Y gene homologues confirms these layers [29]. The oldest region of the XCR (stratum 1), corresponds to the evolutionary block on chicken chromosome 4, and the next oldest region (stratum 2), corresponds to the block on chicken chromosome 12. In humans, the XAR is subdivided into two regions (strata 3 and 4) that were prevented from recombining with the Y by major rearrangements (probably large inversions on the Y). The oldest stratum of the X contains *SOX3*, the ancestor of *SRY*, consistent with the hypothesis that the evolution of this gene on one member of the ancestral autosome pair defined the sex chromosomes of therian mammals.

Although most X genes are not involved in sex-related functions, there is bias toward reproduction related genes [30]. This may be the result of hemizygosity in males, as recessive alleles are instantly open to selection on the single X in males, so male-advantage genes are predicted to accumulate on the X. This is consistent with the finding that X genes are over-represented among genes expressed in mouse spermatogonia [31], and the number of human X-linked gonadal abnormality syndromes.

X-linked gonadal dysgenesis is frequently accompanied by mental retardation, and it appears many X genes have evolved functions in both the brain and the gonad. Intelligence genes (that is, the genes whose mutation causes mental retardation) are represented in excess on the human X [32]. The mammalian X has been proposed as the "engine of speciation" as the accumulation of sex and intelligence genes may have created pre- and post-mating reproductive isolation [33].

The loss of genes from the Y chromosome creates unpartnered X genes that are present in only one copy in males, so are under-expressed in comparison with autosomal genes. This dosage disparity is thought to have selected strongly for a dosage compensation scheme. X genes are up-regulated twofold, equalizing the gene dosage between the X and the autosomes in males. In females, such up-regulation would lead to overexpression of X genes, so one X in females is transcriptionally silenced, a process known as X chromosome inactivation (XCI). XCI results from a complex hierarchy of chromatin silencing changes, and is controlled by the *XIST* locus in placental mammals. In marsupials, XCI appears to be a simpler and less stable system. *XIST* is not present in marsupials and was assembled relatively recently in the placental lineage [34, 35].

The acquisition of a sex-determining locus has therefore led to substantial differentiation of the therian X and Y from an ordinary autosome pair. These complex evolutionary processes were initiated by a single event, the evolution of the *SRY* gene and its acquisition of the role as sex-determining trigger.

## *SRY* – Gene structure and function

The human testis-determining factor (TDF) is a dominant male factor that initiates formation of a testis in XY, and even XXY embryos (Klinefelter's syndrome) [36]. In XX and XO embryos (Turner's syndrome) no testis is initiated, and the genital ridge differentiates into an ovary [37].

Identification of the testis-determining gene on the Y resulted from analysis of regions of the Y transferred to the X in XX males. A gene (*ZFY*) was first proclaimed to be TDF [38], but was discounted first when it was discovered to be autosomal in marsupials [39], and shortly after was excluded from the minimal TDF region in XX males [40]. The gene *SRY* was subsequently isolated from a region of the Y close to the PAR, and mutation analysis of XY females indicated that *SRY* was responsible for sex determination [41, 42]. This was confirmed by the generation of XX *Sry*+ male transgenic mice [43]. An *SRY* orthologue has been identified in all other placental mammals investigated (with a few exceptions that have lost *SRY*) [2–4], and the conserved function of *SRY* as the testis-determining gene has been inferred by the male development of XX male transgenic mice harboring a human or goat *SRY* transgene [44, 45].

*SRY* orthologues have also been identified in marsupial species [46], indicating that *SRY* was present in the therian lineage before the divergence of marsupials and placentals. *SRY* maps to the tiny marsupial Y chromosome. Whereas *SRY* expression is largely restricted to the brain and testis in placental mammals, in tammar wallaby its expression is nearly ubiquitous [47]. In the absence of mutants and knockouts/transgenics, it has not been possible to demonstrate directly that *SRY* is sex determining in marsupials. There is another candidate for the marsupial sex-determining switch. In marsupials (but not placentals), the sex-reversing *ATRX* gene on the X has a Y-borne partner, *ATRY*, which is appropriately expressed in the testis [48].

The first action of *SRY* as the male sex determinant may be to promote cell proliferation. *Sry* has been found to induce cell division in mouse fetal gonads

immediately prior to testis differentiation [49], and the accelerated growth of males compared to females has been noted in preimplantation embryos of mice, humans, and cows (reviewed in [50]). The male-specific proliferation of cells in the gonad is critical for testis determination, as disruption of this process results in male-to-female sex reversal [49]. The first histological marker of the testis is the differentiation of the Sertoli cells, initiated by the expression of *SRY.* This is observed clearly in mice where *Sry* expression in the developing gonad is up-regulated in a brief spike in the pre-Sertoli cells between 10.5 and 12.5 day post coïtum (dpc) immediately preceding Sertoli cell differentiation; each cell may be exposed to *Sry* activity for 8 h or less [51, 52].

In humans *SRY* expression also surges in the male gonad immediately prior to Sertoli cell and testis determination, and expression persists in the testis into adulthood [53]. Similarly dog, sheep and pig *SRY* is up-regulated during the critical window of sex determination; *SRY* expression is maintained in the adult testis in dog and sheep but down-regulated in pig [54–56]. A male-determining signal initiated by *SRY* could be maintained epigenetically by virtue of its interaction with *KRAB-O* and associated chromatin remodeling factors [57].

An additional role for *SRY* in the brain has been intensively debated because of the possibility that this gene may be responsible for sex differences in behavior. *SRY* transcription has been reported in the brain of rodents and humans, but no difference in mating behavior has been noted in XX male sex-reversed mice [58]. Specific *Sry* knockdown in the brain of rats, however, results in loss of coordination [59].

The activity of *SRY* is thought to be mediated through a conserved DNA-binding domain called the HMG box, first observed in high mobility group proteins. The HMG box is the only region of the gene conserved between species [46], and all but a few sex-reversing mutations cluster within this region (reviewed in [60]). The *SRY* HMG box binds with high affinity at the consensus sequence (A/T)ACAA(T/A) and bends DNA through a specific angle, which is critical for its function [61]. Sex-reversing mutations within the *SRY* HMG box cause impaired binding and DNA bending function. *SRY* may therefore act as an architectural factor to control transcription. Consistent with a role in gene regulation, *SRY* is imported into the nucleus, and importin-β and calmodulin have been demonstrated to play a role in its translocation *in vitro* [62]. *SRY* has been shown to both activate and repress reporter constructs *in vitro* [63–65]. In particular *SRY* is proposed to up-regulate the testis-determining gene *SOX9.* The up-regulation of *SRY* in pre-Sertoli cells is closely followed by the up-regulation of *SOX9* expression, a gene capable of initiating successful testis development in the absence of *SRY* [66, 67].

Although *SRY* has been generally supposed to be a transcription activator, the poor conservation of this gene (outside of the HMG box *SRY* orthologues cannot be aligned) suggested that it might act, instead, as a repressor of a repressor [68]. *SRY* was proposed to function as a repressor in a double inhibition of a putative factor Z to explain rare familial XX male syndromes [69]. The putative intermediate gene was later proposed to be the X-borne gene, *SOX3*, from which *SRY* evolved [70].

## The evolution of *SRY* from *SOX3*

The closest relative of *SRY* is *SOX3,* found on the X chromosome in all therian mammals and proposed to be the gene from which *SRY* evolved [71]. *SOX3* belongs to a family of genes that share the *SRY* HMG box DNA binding domain, for which they were named *SOX* (*SRY*-like HMG box). *SOX3* mutations result in mental retardation, growth hormone deficiency and failure of spermatogenesis, although not sex reversal [72–74].

*SOX3* is expressed in the indifferent gonad in humans and mice [27, 75], but not marsupials [76]. However, the expression of *SOX3* in the developing gonads of *Xenopus* and chicks [77, 78] suggests that *SOX3* expression in the urogenital ridge is a conserved vertebrate trait that has been subsequently lost in marsupials.

It has been suggested that a truncation mutation of *SOX3* resulted in a dominant suppressor that assumed the role of the male sex-determining switch in the same way that truncation of the related *SOX* gene, *SOX9,* results in dominant suppression [71, 79]. The evolution of *SRY* presumably defined the therian X and Y as sex chromosomes; a mutation of one *SOX3* allele on the autosome pair that was the ancestor of the therian X and Y would have defined the proto-Y, whereas the other copy of *SOX3* remained unaffected on the partner chromosome, the proto-X.

Non-mammalian vertebrates have no orthologue of *SRY,* and *SOX3* is autosomal, consistent with a causative role for *SRY* in the differentiation of the therian XY pair. It has been thought, therefore, that the evolution of *SRY*, and the beginning of XY differentiation in mammals, must have occurred shortly after the divergence of mammals and reptiles 310 MYA. However, recent studies of sex and *SRY* in the most basal mammals, the egg-laying monotremes, have completely changed our perspective on when and how this occurred.

## Sex chromosomes of monotreme mammals

Monotreme mammals provide an evolutionarily intermediate between therian mammals and reptiles (including birds) to further pinpoint when and how *SRY* evolved. Although debate continues about just when monotremes diverged from other mammals, there is now no doubt that they diverged from placentals earlier than marsupials (reviewed in [80]), and this has recently been confirmed by whole genome sequencing of the platypus [12]. There are only five extant monotreme species in two families: the platypus (*Ornithorhynchus anatinus*), and four echidna (spiny anteater) species, the short-beaked echidna (*Tachyglossus aculeatus*), and three closely related long-beaked echidnas (*Zaglossus bartoni*, *Zaglossus attenboroughi*, *Zaglossus bruijni*). Platypuses and echidnas diverged about 64 MYA.

The sex chromosome system of monotremes, in addition to being important in comparative analysis, has been of long-standing interest in its own right. Several unpaired chromosomes in males signified the presence of multiple X and Y chromosomes [81], and the presence of a chain of several chromosomes at meiosis signified that sex chromosomes were part of a translocation complex [82]. The advent of chromosome painting technology enabled recognition in male platypus of five X ($X_1$, $X_2$, $X_3$, $X_4$ and $X_5$) and five Y chromosomes ($Y_1$, $Y_2$, $Y_3$, $Y_4$ and $Y_5$) which together form a ten member chain in the order $X_1$, $Y_2$, $X_2$, $Y_2$, $X_3$, $Y_3$, $X_4$, $Y_4$, $X_5$, $Y_5$ during male meiosis [15, 83]. The sex chromosomes then segregate in an alternating pattern to ensure balanced gametes, with female-determining sperm carrying all five X chromosomes and male-determining sperm carrying all five Y chromosomes. An analogous situation occurs in the echidnas, with the short-beaked species possessing five X ($X_1$, $X_2$, $X_3$, $X_4$ and $X_5$) and four Y chromosomes ($Y_1$, $Y_2$, $Y_3$ and $Y_4$), which together form a nine member chain in the order $X_1$, $Y_2$, $X_2$, $Y_2$, $X_3$, $Y_3$, $X_4$, $Y_4$, $X_5$ during male meiosis [11]. The number of chromosomes in the monotreme meiotic chains is unprecedented amongst vertebrates; meiotic multiples of more than four are found only in plants (*e.g.*, the classic evening primrose) and invertebrates species (*e.g.*, huntsman spiders). The chain has been thought to be the product of successive reciprocal translocations, or alternatively could have resulted from the hybridization of populations with differing Robertsonian translocations [82–85]. The platypus and echidna chains comprise mostly homologous elements, but, surprisingly, appear to have independently incorporated one different autosome into their sex-linked chains [11]. Cross-species hybridization experiments show that platypus $Y_3$, $X_4$ and $Y_4$q correspond to the short-beaked echidna chromosome 27, and the short-beaked echidna $Y_4$q and $X_5$q correspond to platypus chromosome 11. The most parsimonious explanation is that these autosomes were incorporated after the divergence of platypus and echidna. This favors a genesis model using successive reciprocal translocations, rather than the hybridization of populations with differing Robertsonian translocations [11].

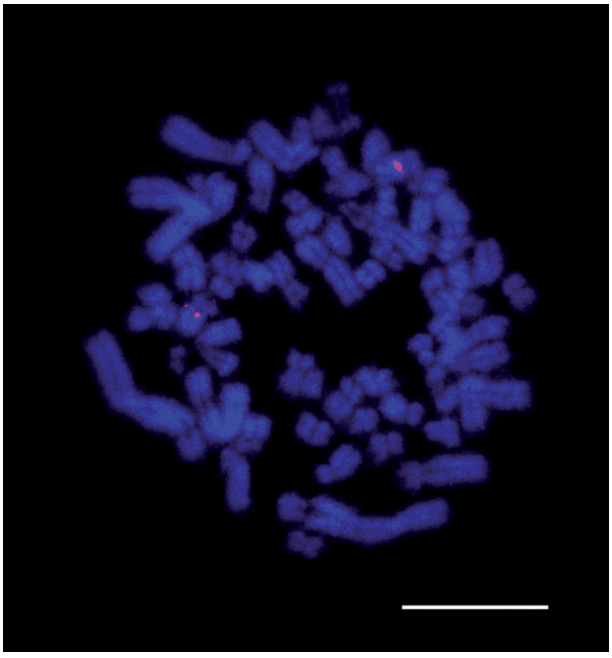

**Figure 2.** Fluorescence in situ hybridization of *SOX3* to short-beaked echidna chromosome 16, the homologue of platypus chromosome 6 (to which *SOX3* has also been mapped, inferring the autosomal localization of *SOX3* in the ancestral monotreme). Scale bar represents 10 μm.

## Is *SRY* the sex-determining gene in monotremes?

Over many years an orthologue of *SRY* has been sought in the monotremes. Southern blots never identified a male-specific HMG box gene in monotremes, and screening monotreme genomic or testis cDNA libraries also produced no male-specific *SRY*-like genes. Likewise, PCR using many combinations of primers failed to elicit a male-specific product. It appeared that there was no *SRY* homologue in platypus or echidna, but a divergent *SRY* gene could not be ruled out [15].

The monotreme $X_1$ is conserved between platypus and echidna, and was originally reported to have some homology to the therian X [86]. This is now known not to be the case. The entire conserved region of the

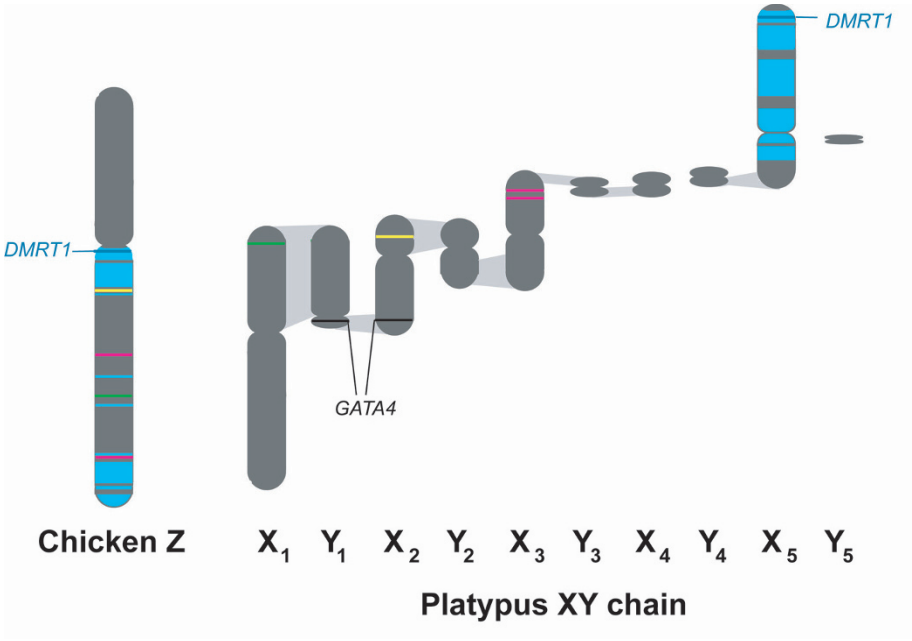


**Figure 3.** Putative sex-determining genes and homology between the platypus sex chromosomes and bird Z. Inferred pseudoautosomal regions of the platypus sex chromosomes are shaded in light gray.

therian X, including *SOX3* (the *SRY* homologue), was mapped to platypus chromosome 6, and there is no homology between the monotreme and therian sex chromosomes [10–13, 87]. *SOX3* was also mapped to the homologous autosome in the short-beaked echidna (Fig. 2). This dates the origin of *SRY*, and the birth of the therian X and Y, to a very narrow window between the divergence of monotremes and therians 166 MYA, and the divergence of marsupials and placentals 148 MYA, much more recently than previously thought.

Unexpectedly, orthologues of genes on the platypus sex chromosome map, not to the therian sex chromosomes, but to bird sex chromosomes [11–13, 15, 16]. Figure 3 shows the considerable homology between the chicken Z and platypus chromosomes $X_1$, $Y_1$, $X_2$, $X_3$, and especially $X_5$. It was a surprise to find that the monotreme sex chromosomes share homology to the sex chromosomes of birds, which have a female heterogametic system and a most recent common ancestor with monotremes 310 MYA. The homology between the monotreme and avian sex chromosomes raises the previously unconsidered possibility that a sex chromosome system ancestral to all amniotes is retained in birds and monotremes. The mapping of orthologues from the human X and bird Z to the same autosomal linkage group (ALG2) in the amphibian salamander (Ambystoma) is therefore particularly intriguing [88].

To understand how *SRY* took over a sex-determining role, it is important to discover how sex was determined at the base of the mammals. Identification of a sex-determining gene in monotremes will provide clues to the identity of the ancestral mammal sex-determining gene.

## Does a bird Z-borne gene determine sex in monotremes?

The recent discovery that gene orthologues from the bird Z chromosome map to the monotreme X chromosomes suggests that birds and monotremes might share an ancient sex-determining gene. It is still far from clear how sex is determined in birds, and even whether there is a female-dominant gene on the W, or a dosage-dependent gene on the Z, or both. The sexual phenotype of chicken ZZW triploids indicate that birds may have a W-linked female factor in addition to a Z-linked male sex-determining gene [89]. ZZW triploid chicks begin to develop as phenotypic females but mature as males. Investigations into bird sex determination have been hampered by the absence of bird mutants, transgenics and sex chromosome aneuploids (predicted to be lethal because of inappropriate dosage compensation [90]).

An orthologue of a putative bird sex-determining gene on the Z chromosome, *DMRT1* [91, 92], is an obvious candidate for the monotreme sex-determining gene. *DMRT1* maps to the Z but not the W in all bird species, even the basal ratites (large flightless emus and ostriches), in which most of the W is shared with the Z [93]. Unlike the male dominant activity of *SRY*, *DMRT1* is proposed to act in a dosage-dependent manner to initiate male development in ZZ bird embryos (which have two copies) but not ZW embryos

(which have only one copy). *DMRT1* is expressed in the bipotential gonad of chickens, then up-regulated in the testis, and down-regulated in the ovary, consistent with a function in male sex determination [91]. A role for *DMRT1* in testis development is not limited to birds; *DMRT1* is also up-regulated in the embryonic testis of mice, humans and alligators [91, 92, 94]. Furthermore, deletions of the terminal region of human chromosome 9 that bears *DMRT1* have been implicated in gonadal dysgenesis and male to female sex reversal [95]. A sex-differentiating function may even be conserved in invertebrates, as *DMRT1* shares homology with the *Drosophila melanogastor doublesex* gene and the *Caenorhabditis elegans mab-3* gene, both of which are involved in sexual development (hence the name *DMRT1*, *doublesex* and *mab-3*-related transcription factor 1).

Could *DMRT1* act as the sex-determining switch in monotremes? *DMRT1* is located on platypus $X_5$ and echidna $X_4$, which represent homologous chromosomes in the chain, and there is no detectable copy on a Y [11, 15]. *DMRT1* is therefore present in a higher dose in monotreme females than in males, the opposite dosage relationship from that in the bird ZZ male/ZW female system. One possibility is that the X-linked copies of *DMRT1* are dosage compensated in females, implicating dosage compensation as a sex determination device. Indeed, X inactivation has been suggested to have originally evolved as a sex-determining mechanism, rather than a mechanism to correct dosage discrepancy [96]. However, this model requires that the gene has copies on the Y as well as the X: one X allele is silenced by X inactivation in females, while both the X and Y copies escape regulation in males, thus creating a 2:1 dosage discrepancy in favor of males. This does not appear to be the case for platypus or echidna, however, as no *DMRT1* has been identified on a Y. X inactivation may therefore correct the *DMRT1* dosage imbalance, but it is not clear how the balance could be tipped in favor of males.

Although *DMRT1* seems unlikely to function as the monotreme sex-determining switch, it is still likely to be up-regulated during male differentiation, as in other vertebrates. The single copy of *DMRT1* may be up-regulated in males by a downstream compensatory mechanism. This has been shown to occur in ZW male chickens sex-reversed with an aromatase inhibitor; these birds up-regulate the single *DMRT1* allele and achieve successful testis differentiation [97]. In the adult platypus (embryonic tissue is not available), *DMRT1* expression is confined to the testis [16], consistent with a role in testis function.

If *DMRT1* is not the sex-determining factor in monotremes, does another chicken sex-determining gene have this function? Several other putative sex-determining factors have been described on the chicken W that may have homologues on the platypus X that act in the female pathway.

One of the first genes described on the W was *HINTW* (histidine triad nucleotide binding protein, W-linked; also known as *WPKCI* or *ASW*), which is present in approximately 40 copies on the chicken W. A single homologue *HINTZ* is located on the Z, a situation parallel to the many amplified sex-specific genes on the mammal Y that evolved from a single copy on the X. *HINTW* has high expression in female embryonic gonads, and may function in a dominant-negative manner by heterodimerizing and inhibiting *HINTZ* [98, 99].

A sex-specific role for *HINTW*, however, is likely to be limited to the carinate birds. In ratites, *HINT* lies within the large pseudoautosomal region, with a single copy on both the Z and W [98]. The absence of sex-specific *HINT* alleles in ratites, and lack of dosage difference between males and females, would exclude *HINTW* from a conserved role in initiating the evolution of the bird ZW. A role in monotreme sex determination therefore seems unlikely.

*FET-1* (female-expressed transcript 1) was isolated from a screen for genes expressed differentially in the male and female chick gonad [100]. *FET-1* is expressed highly, and nearly exclusively, in the ovary immediately before and during differentiation. *FET-1* is present in a single copy on the W, and no homologue has been identified on the Z. However, no *FET-1* orthologues have been identified in other birds, and *FET-1* may represent a recently retrotransposed retroviral element in the chicken [101]. Thus, the presence of this gene on a monotreme sex chromosome is unlikely.

In the absence of obvious candidate sex-determining genes from the bird Z and W, we turned to the other components of the platypus sex chromosome complex.

## Candidate genes from the platypus Y chromosomes

The five Y chromosomes of the platypus are the obvious place to look for sex-determination genes. These multiple Ys present a large target, constituting about 6% of the haploid genome, although much of the terminal regions of these chromosomes have homology to, and pair with, the adjacent X chromosomes in quite extensive pseudoautosomal regions. The $Y_5$ is perhaps the most likely repository of critical sex-determining information, since this tiny chromosome proves to have been transposed to other sex chromosomes in the echidna [11].

**Table 1.** Genes in the sex-determining pathway and their locations in human (HSA), chicken (GGA) and platypus (OAN). Genes that are sex-linked in platypus are shaded.

| Gene | HSA location | GGA location | OAN location |
|---|---|---|---|
| WNT4 | 1p35 | Chr21 6.5Mb | Chr5q |
| RSPO1 | 1p34.3 | Chr23 3.5Mb | Chr16 |
| LHX9 | 1q31-q32 | Chr8 1.3Mb | Chr4q |
| FOXL2 | 3q23 | Chr9 6.8Mb | Contig10930 11Kb |
| PDGFRA | 4q11-q13 | Chr4 67.3Mb | Contig8283 7kb |
| PGDS | 4q22.3 | Chr4 36.6Mb | Ultra445 8.5Mb |
| POD1 | 6q23.1 | Chr3 58.2Mb | Ultra520 2.5Mb |
| HOXA10 | 7p15-p14 | Chr2 31.5Mb | Ultra231 2.3Mb |
| GATA4 | 8p23.1-p22 | Chr3 110Mb | $ChrX_1/Y_1$ |
| FOG2 | 8q23 | Chr2 136Mb | Chr8 37Mb |
| DMRT1 | 9p24.3 | ChrZ 26.7Mb | $ChrX_5q$ |
| NR5A1 | 9q33 | Chr17 10.1Mb | Chr4q |
| PAX2 | 10q24 | Chr6 18.1Mb | Chr17 1.3Mb |
| EMX2 | 10q26.1 | Chr6 24.6Mb | Ultra272 8.4Mb |
| WT1 | 11p13 | Chr5 4.5Mb | Chr3p |
| DHH | 12q12-q13.1 | ? | Ultra148 0.16Mb |
| AMHR2 | 12q13 | ? | ? |
| FGF9 | 13q11-q12 | Chr1 179.6Mb | Chr20q |
| LGR8 | 13q13.1 | Chr1 179.15Mb | Ultra336 0.49Mb |
| SOX8 | 16p13.3 | Chr14 5Mb | ? |
| LHX1 | 17q12 | Chr19 8.3Mb | Chr17/18q |
| SOX9 | 17q24.3-q25.1 | Chr18 8.5Mb | Chr15p |
| CBX2 | 17q25.3 | Chr18 9.6Mb | Contig13951 18Kb |
| AMH | 19p13.3-p13.2 | Chr28 1.6Mb | Contig22983 9Kb |
| INSL3 | 19p13.2-p12 | ? | ? |
| NROB1 | Xp21.3-p21.2 | Chr1 119.2Mb | Chr15q |
| ARX | Xp21 | Chr1 121.29Mb | ? |
| ATRX | Xq13 | Chr4 12.4Mb | Chr6 11.1Mb |
| SOX3 | Xq27.1 | Chr4 10.6Mb | Chr6 |

Since a female platypus was sequenced [12], there is no Y sequence directly available. However, many genes have now been mapped to the X chromosomes, and several of these have Y homologues. Although these pseudoautosomal genes, themselves, are unlikely to act in sex determination, they define the potential gene content of the Y chromosomes in the differentiated regions. Therefore, possible candidates for the platypus sex-determining switch may be identified by searching the X sequence for homologues of genes involved in sex differentiation in other vertebrates.

The platypus genome sequence assembly [12], along with BACs anchored to the platypus sex chromosomes [11, 13, 15, 102], have revealed large regions of homology to chicken chromosomes 2, 3, 12, 13, 16, 17 and Z, which represent parts of human chromosomes 2, 5, 6, 8, 9 and 18. Scanning the platypus genome assembly for genes involved in the vertebrate sex-determining pathway (reviewed in [103]) reveals that, with the exception of *DMRT1* and *GATA4*, all map to autosomes or unanchored scaffolds (Table 1).

In addition to *DMRT1,* discussed above, the sex pathway gene *GATA4* also maps to the platypus sex chromosomes. *GATA4* maps to the PAR of platypus $X_1$ and $Y_1$, and so is present in two copies in both males and females [102]. *GATA4* is a member of a family of zinc-finger transcription factors which recognize the consensus target sequence T/A(GATA)A/G, and is proposed to promote male development. *Gata4* is up-regulated in mouse Sertoli cells, and therein the interaction of *Gata4* and *Fog2* (friend of *Gata2*)

promotes *Sry* expression [104]. *Gata4* is down-regulated in the differentiating mouse ovary. The male-determining function of *GATA4* may be limited to therian mammals, however, as sexually dimorphic *GATA4* expression is not observed in the chicken developing gonad [105]. *GATA4* maps to human chromosome 8, and is present on platypus $X_1$ and $Y_1$ amongst many other gene homologues from a syntenic block of human chromosome 8 and chicken chromosome 3. A sex-determining role for *GATA4* would require allelic differences or a dosage discrepancy between males and females; this could be mediated by X inactivation or another form of dosage compensation. Dosage compensation within the PAR has been observed for at least one human gene (*SYBL1*) [106]. Searches for sex-linked alleles of *GATA4*, and for dosage differences on $X_1$ and $Y_1$ will be important.

Thus far, then, the only candidate genes for platypus sex determination worth further consideration are *DMRT1* and *GATA4*.

## Sex determination in the ancestral mammal

*SRY* and the therian X and Y are clearly recently derived, having their origin between the divergence of monotremes from therians 166 MYA and the marsupial-placental split 148 MYA. What was the sex-determining system of an ancestral mammal from which this new system sprang?

There is a wide variety of sex-determining systems in reptiles and birds, including environmental and genetic systems (and a combination of the two). Amongst genetic systems, there are many with differentiated sex chromosomes, but none of these have any homology with therian sex chromosomes. This variety, and the lack of homology with therian sex chromosomes has discouraged speculation about an ancestral amniote system

The unexpected homology of the monotreme sex chromosomes with the bird Z, however, immediately poses the question: do the sex chromosomes of birds and monotreme mammals have a common origin?

The ancestor of amniotes was originally suggested to determine sex by temperature because of the wide distribution of this system throughout the clade. The numerous amniote genetic systems could all have been independently derived from it, as was suggested for the mammal XY and bird ZW [7]. However, recent work on the dragon lizard *Pogona vitticeps* that combines both ZZ/ZW sex chromosomes and temperature sex determination [107] provides a mechanism by which environmental and genetic sex-determining systems are interconvertible and easily selected by changes in environmental conditions. Temperature and genetic sex determination may coexist throughout reptiles, and it has been speculated that many reptiles with temperature sex determination may also have an underlying genetic mechanism. It is possible that the ancestral amniote combined sex chromosomes and temperature sex determination, which could explain the widespread prevalence of temperature sex determination in reptiles.

A sex chromosome system ancestral to amniotes would be expected to be widely represented in birds, mammals and reptiles. Supporting this hypothesis is the observation that the bird Z chromosome is highly conserved between species, even including ratites [108] and the karyotype is extremely stable, being largely shared by turtles [7]. However, snakes have an unrelated ZW system with homology to the bird chromosome 2 rather than the Z [6]. Other turtle and lizard species have XY or ZW sex microchromosomes, which bear no resemblance to the bird sex chromosomes [109, 110], although their gene content is not yet known.

Thus, although homology between the avian and monotreme sex chromosomes suggests an ancestral bird ZW-like system in amniote ancestor 310 MYA, the prediction that other reptilian lineages share this ancestral ZW needs to be addressed.

An apparently fatal complication of the hypothesis that bird and monotreme sex chromosome systems are monophyletic is that this implies a switch of sex heterogamety involving the same sex chromosomes, since birds are female heterogametic (ZW) and monotremes are male heterogametic (XY). This has previously been thought to be impossible, or unlikely. However, just such a system has been described in the frog *Rana rugosa* in which homologous XY and ZW chromosomes are maintained [9].

Although it is tempting to try to reconcile a common origin between the sex-determining systems of monotremes and birds, several fundamental questions remain. How did a switch from female to male heterogamety, or *vice versa*, occur? Why is this system not observed in the other reptiles? Was temperature sex determination a component of this ancestral system, or was it subsequently adopted independently in numerous reptile lineages? The possibility that the monotreme and avian sex chromosome systems arose independently remains a viable alternative. Characterizing the sex-determining systems of more reptiles, and searching for homology between the sex-determining systems of reptiles and amphibians will assist in reconstructing the ancestral state.

## Conclusion

Comparisons amongst vertebrates have revealed a variety of sex-determining mechanisms, surprising for such a vital function. Whereas genes downstream in the sex-determining pathway appear to have been conserved across vertebrates, the sex-determining trigger shows extreme variation between vertebrate taxa. Comparative studies, particularly of the basal monotreme mammals, shows that the therian sex-determining gene, *SRY,* is a relatively new male dominant sex-determining allele. Thus the evolution of *SRY* from the autosomal *SOX3* gene 166–148 MYA initiated the differentiation of a new X and Y sex chromosome pair early in therian evolution. This re-dating of the origin of the XY system of therian mammals necessitates a recalculation of the rate at which the human Y chromosome has degraded, and reduces the predicted lifetime of the human Y [14]. The evolution of *SRY* corresponds with the divergence of the therian and monotreme lineages, raising the possibility that the genesis of *SRY* provided the reproductive isolation that separated these two mammal subclasses. In mole voles the modification of the sex-determining switch, in this case involving the loss of *SRY*, was also concurrent with speciation, and the two Y-less species are also separated by variations in their X chromosomes [111]. Similarly, a new sex-determining allele, *DMY*, was derived from *DMRT1* immediately before the speciation of the medaka fish *Oryzias latipes* and *O. curvinotus* [112]. The evolution of a new sex-determining gene may initiate a chain of events that leads to irreversible reproductive isolation.

Interest in elucidating the sex-determining system from which *SRY* assumed control in therians has intensified following our recent finding that the sex chromosomes of birds and monotremes share homology. The possibility that the ancestor of amniotes harbored a sex chromosome system still maintained in monotremes and birds today, while intriguing, still faces several obstacles: the apparent lack of homology between many amniote sex chromosomes, the frequency of subsequent transitions to temperature sex determination in reptiles, and an inferred sex heterogamety transition using the same sex chromosomes. The search for the monotreme sex-determining mechanism is of fundamental importance, as it has implications not only to our understanding of their curious multiple translocation system, but also the evolution of sex determination in mammals and perhaps amniotes.


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