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Sex chromosomes and sex determination in reptiles

Commentary

William S Modi[1] and David Crews[2]

Reptiles occupy a crucial position with respect to vertebrate phylogeny, having roamed the earth for more than 300 million years and given rise to both birds and mammals. To date, this group has been largely ignored by contemporary genomics technologies, although the green anole lizard was recently recommended for whole genome sequencing. Future experiments using flow-sorted chromosome libraries and high-throughout genomic sequencing will help to discover important findings regarding sex chromosome evolution, early events in sex determination, and dosage compensation. This information should contribute extensively toward a general understanding of the genetic control of development in amniotes.

Addresses
[1] SAIC Frederick, National Cancer Institute, Core Genotyping Facility, 8717 Grovemont Circle, Gaithersburg, MD 20877, USA
[2] Section of Integrative Biology, University of Texas, Austin, TX 78712, USA

Corresponding author: Modi, William S (modiw@mail.nih.gov)

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Introduction

Reptiles are a familiar group of vertebrates, having existed for more than 300 million years. Although these animals reached their zenith during the Jurassic and Cretaceous periods, today they are represented by only four orders (turtles, crocodilians, squamates [snakes and lizards] and sphenodontians [tuatara]) (Figure 1). However, not only do these animals occupy a pivotal position on the phylogeny of vertebrates — they are the direct ancestor to birds and mammals — but they also possess several unique biological attributes that, if better understood, could contribute significantly to understanding basic evolutionary biology and the molecular mechanisms behind human health and disease. Recent technical advances in DNA sequencing have made whole genome sequencing possible for a variety of species. One mission of the ambitious National Human Genome Research Institute (NHGRI) is whole genome sequencing of various animal species. Among vertebrates, whole genome sequences are now available for chicken, *Xenopus* and three species of fish, and the sequences of over 20 species of mammals are complete or underway (http://www.genome.gov/11007951). Interestingly, reptiles have remained impervious to the watchful eye of the NHGRI's comparative sequencing program. However, at NHGRI's request, the Reptile Genome Consortium met in April 2005 at Washington University's Genome Sequencing Center in St Louis. Their subsequent mission was to consult the scientific community and make recommendations to the Institute on which species should be considered for whole genome sequencing. Their recommendation was submitted to NHGRI as a 'White Paper' in July 2005, and the community overwhelmingly chose the green anole lizard, *Anolis carolinensis*, as its first target species, with the American alligator, garter snake and/or painted turtle to follow (http://reptilegenome.com). The green anole is an excellent choice, having been used as a model system for reptilian physiology, neurology and reproductive behavior for many years [1].

In addition to the whole genome sequencing of *Anolis* and subsequent species, parallel investigations into reptilian genomics will unlock many of the fascinating secrets that nature has bundled into these intriguing animals. This review summarizes research on reptilian sex chromosomes and sex determination, and recommends the preparation of flow-sorted chromosome-specific libraries (see Glossary). Such libraries will enable reconstruction of the evolutionary events involved in sex chromosome diversification and will provide the raw materials necessary to study the expression of specific genes involved in sex determination and dosage compensation.

Has the mechanism of sex determination evolved independently in different reptilian lineages?

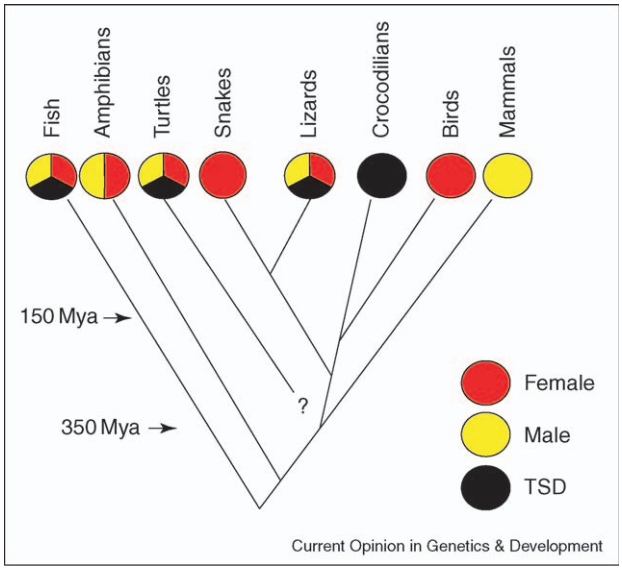
Sex chromosomes are distinct from autosomes in that they differ in size, number, staining characteristics, and gene content when the two sexes are compared. Ohno's law [2] asserts that heteromorphic sex chromosomes originated from an autosomal ancestor following a mutation that conferred a sexual advantage. Additional sex-linked mutations in other genes then accumulated on the same homologue. Recombination between the primordial sex chromosomes was suppressed by chromosomal rearrangements such as inversions to preserve the block of sex-linked genes. The absence of recombination fostered the accumulation of mutations and repetitive sequences with subsequent 'heterochromatization' of the sex-specific chromosome. Deletions of heterochromatin account for the smaller sizes usually observed for the Y or W chromo-

Glossary
Boids: The snake taxonomic family Boidae contains boa constrictors and pythons.
Diploid–triploid somatic mosaicism: The presence of different chromosome numbers in different adult tissues of the same individual. In this case, certain tissues have a diploid (2n) complement, whereas other tissues have a triploid (3n) complement.
Flow-sorted chromosome-specific libraries: A sample of genomic DNA enriched for a specific chromosome, prepared by separating a suspension of mixed chromosomes with fluorescence-activated cell sorter.
Male- or female-permissive temperature: The ambient temperature that causes embryonic development of a bipotential gonad into a testis or ovary in species with temperature-dependent sex determination.
Subtractive hybridization analyses: A molecular biological procedure that compares RNA levels from different tissues in an attempt to identify transcripts that are over- or under-expressed in one tissue relative to in others.
Viperids: This family of poisonous snakes contains Old World vipers, such as puff adders, bushmasters and sand vipers.

somes compared with the X or Z chromosomes, respectively.

Reptiles exhibit some of the most extraordinary variability in sex chromosome structure and patterns of sex determination seen among vertebrates (Figure 1) [3]. For example, all crocodilians, the tuatara, most turtles and many lizards have temperature-dependent sex determination (TSD), in which adult anatomical sex is a function of the temperature at which eggs are incubated. Species that display TSD do not reveal karyotypic differences between males and females, and the range in temperature that produces all males or all females can be as little as 1 °C [4].

Figure 1



Vertebrate phylogeny illustrating sex determination modes in different taxa. ''Female'' and ''Male'' represent genetic sex determination with female and male heterogamety, respectively. TSD represents temperature-dependent sex determination. An unanswered question in contemporary reptilian phylogenomics regards the relationships of turtles to other reptiles.

By contrast, several turtles, some lizards and all snakes are subject to genetic sex determination (GSD), in which adult sex is chromosomally determined at the time of fertilization. At least two species of turtles and some lizards have male heterogamety (XY males and XX females), whereas other turtles, other lizards and all snakes have female heterogamety (ZZ males and ZW females). Other turtles are chromosomally monomorphic, and additional experiments are needed to determine if they have GSD or TSD. The ZW chromosomes of snakes reveal increased differentiation as one progresses from the phylogenetically primitive boids to the more advanced viperids (Figure 2a; see also Glossary). The origin of heteromorphic XY sex chromosomes in two species of turtles is thought to have occurred independently, and these same chromosomes appear as autosomes in other species of turtles with TSD (Figure 2b) [5,6].

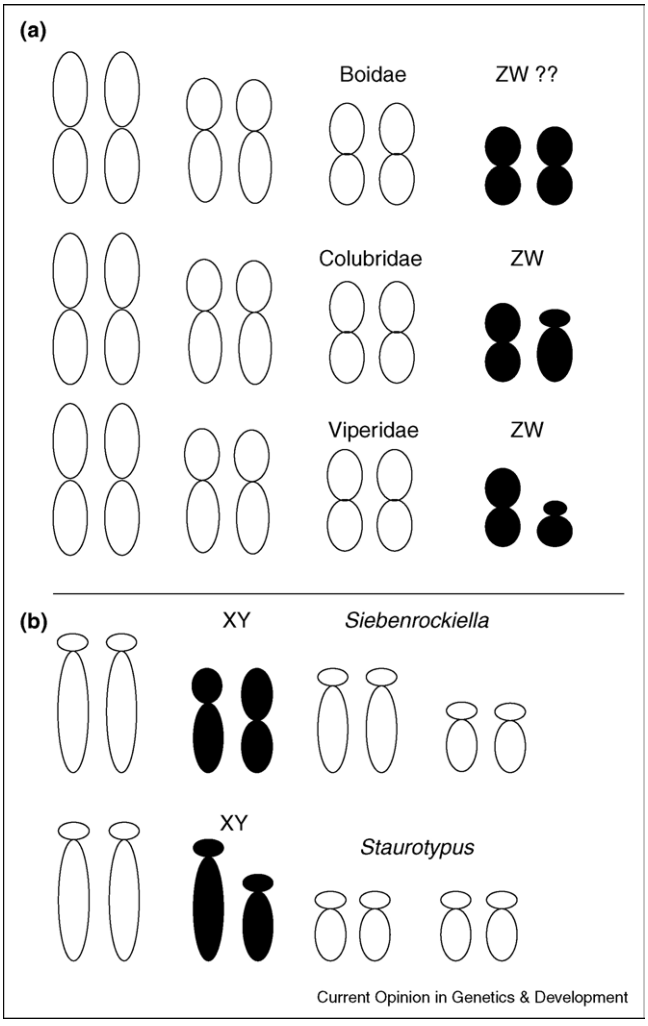
The variability seen among reptilian sex chromosomes suggests that sex chromosome and sex determination systems have evolved independently in different lineages. However, definitive molecular cytogenetic and gene mapping data for reptilian sex chromosomes, which would precisely define their evolutionary histories, are lacking. An important set of experiments would use flow cytometry [7] to prepare flow-sorted W, X, Y, and Z chromosome-specific libraries from several species with heteromorphic sex chromosomes (Table 1). In an effort to trace the origin of sex chromosome differentiation, these sex chromosome-specific libraries could then be used as hybridization probes in fluorescence *in situ* hybridization to metaphase chromosomes from various species having both TSD and GSD.

Identification of genes involved in vertebrate sex determination

A plausible hypothesis posits that sex-determining systems evolve by the retrograde addition of regulatory elements upstream of established developmental programs [8–11]. If this is accurate, then various sex-determining systems can be thought of as one evolutionarily conserved core network regulated by various taxon-specific upstream factors. For example, many of the same genes that are important in mammalian sex determination are found in other species and show strong similarity in their temporal patterns of expression during gonadogenesis (Figure 3).

Little is known about the cellular and molecular foundations of sex determination in reptiles. The most sought-after but least well-understood are the temperature-transduction mechanism(s) that initiate the TSD

Figure 2



Ideogrammatic depiction of heteromorphic sex chromosomes in two groups of reptiles. **(a)** Chromosomes from three families of snakes, illustrating the progressive differentiation of the ZW system when going from phylogenetically primitive to advanced taxa [2]. **(b)** Chromosomes from two genera of turtles, portraying what are believed to be independently derived XY sex chromosomes in each case [5,6].

pathway. One model species for the study of TSD is the red-eared slider, *Trachemys scripta* [4,12]. Although adult testis and ovary morphologies are highly conserved across amniotes, there are differences in the cellular events involved in gonadogenesis. Two notable differences between mouse and turtle include (i) the mechanism of sex cord formation; and (ii) the initial location and subsequent behavior of primordial germ cells in the gonad [13,14]. In turtles, both males and females form primitive sex cords before sexual differentiation of the gonads. By contrast, mouse testis cords appear to form *de novo* in the male gonad and not at all in the female gonad [15]. In turtles, primordial germ cells are initially located in the cortex and, subsequently, migrate into cord structures in the medullary region of the gonad at male-permissive temperature, but at female-permissive temperature (see Glossary) remain in the cortex. It is unclear whether recruitment of germ cells to sex cords is driven by supporting cells as it is in mammals or whether germ cells recruit and/or organize the formation of testis cords. Furthermore, the turtle orthologs (*tSox9*, *tWt1*, *tSf1*, *tDmrt1*, *tWnt4*, *tDax1* and *tMis*) of the mammalian genes implicated in sex determination and differentiation are expressed early in the temperature-sensitive period. This suggests that incubation temperature is capable of engaging this core molecular cascade.

Details remain to be elucidated concerning the temporal patterns of gene expression, the cell types that express these genes, and the functions of their gene products. Nevertheless, this model predicts that the significant differences between turtles and mammals are in the upstream regulators, whereas the core downstream pathways mediating ovary and testis development are conserved. There are significant gaps in our knowledge of how temperature acts on target cells in the turtle gonad to influence gene expression, protein activity, mRNA stability and post-transcriptional events. There are similar gaps in our knowledge of mammalian sex determination. In both cases, the identity and action of genes and cells between the initial trigger (genetic or environmental) and the up-regulation of the core sex-determining pathway

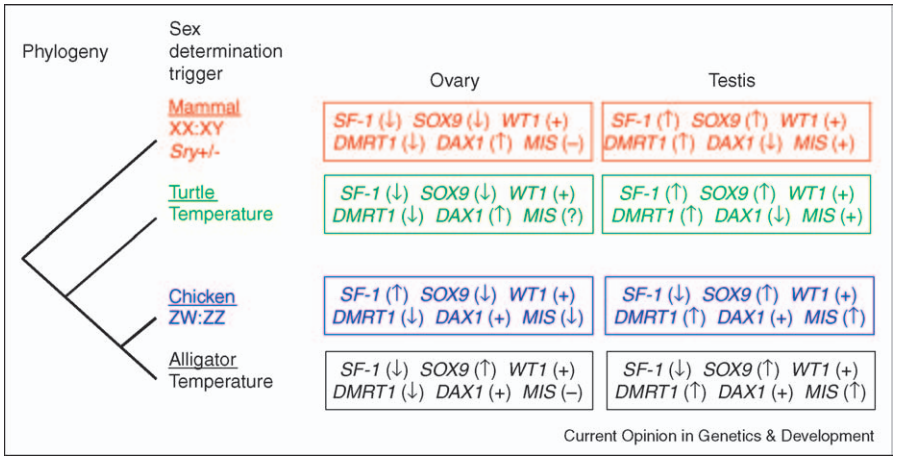
Table 1

Species of reptiles with heteromorphic sex chromosomes that could be used to generate flow-sorted, chromosome-specific, genomic DNA libraries.

Common name	Scientific name	Mode of sex determination*
Mexican musk turtle	*Staurotypus salvinii*	XX/XY
Asian black pond turtle	*Siebenrockiella crassicollis*	XX/XY
brown-roofed turtle	*Kachuga smithii*	ZZ/ZW
tiger whiptail lizard	*Cnemidophorus tigris*	XX/XY
house gecko lizard	*Gehyra australis*	ZZ/ZW
garter snake	*Thamnophis sirtalis*	ZZ/ZW
Russell's viper	*Daboia russellii*	ZZ/ZW

* XX/XY, refers to genetic sex determination with male heterogameity; ZZ/ZW, refers to genetic sex determination with female heterogameity.

Figure 3



Selected genes underlying differentiation of the genital ridge into an ovary or testis in amniote vertebrates. Phylogenetic relationships indicated on the left of the figure. In mammals and birds, gonadal sex is established by the genetic composition inherited at fertilization, a process known as genotypic sex determination (GSD). In some reptiles, gonadal sex depends, ultimately, on the temperature of the incubating egg, a process known as temperature-dependent sex determination (TSD). The trigger for gonad determination in mammals is the presence (+) or absence (–) of *Sry*; in birds the trigger is unknown but appears to be the Z chromosome to autosome ratio. Note that many of the same genes appear to be involved in gonadal differentiation for species exhibiting GSD (mammals and birds) and TSD (turtles and crocodilians). Note, also, that for these selected genes the patterns of expression appear to reflect phylogenetic relationships, with mammals being similar to turtles, and birds more similar to crocodilians. The regulatory mechanisms behind the expression patterns for most of these selected genes are currently being investigated, but timing of *SOX9* and *MIS* expression during testis development appears to fall along phylogenetic lines; in mammals and turtles, *SOX9* expression precedes *MIS* expression, whereas in alligator and bird the reverse pattern is seen. Finally, through manipulating the genetic, physical or chemical environment it is possible to modify gonadal sex in both GSD and TSD species. Abbreviations: DAX1, dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome; DM, DMRT1, doublesex- and mab3-related transcription factor one; MIS, Müllerian-inhibiting substance; SF-1, steroidogenic factor one; SOX9, SRY-related HMG box nine; SRY, sex-determining region on the Y chromosome; WT1, Wilm's tumor one. Plus (+) indicates presence, and minus (–) indicates absence. Up arrow (↑) indicates up-regulation, and down arrow (↓) indicates down-regulation.

remain obscure. *Trachemys scripta* offers unique advantages and opportunities for experimental manipulation and promises to provide insight into early events of testis and ovary determination.

In organisms subject to TSD, putatively conserved sex-determining pathways are triggered by unknown molecular mechanisms that respond to temperature during the middle third of incubation. Reconstructing the evolution of sex-determining mechanisms is a long-term goal and requires analysis of both conserved and taxon-specific components of vertebrate sex-determining pathways. Although the former is best achieved by examining the roles of known sex-determining genes in a variety of vertebrates, elucidation of mechanisms that are unique to particular sex-determining systems requires *de novo* screening in the organism in question. Subtractive hybridization analyses (see Glossary) have identified genes that differ in their expression in the gonad at male- and female-producing temperatures. Members of this set of promising candidate genes might be involved in sex determination upstream of conserved vertebrate sex-determining genes. Similarly, using a genomics approach, one could determine the nucleotide sequence of entire W and Y chromosomes isolated using flow cytometry. This would produce a list of several hundred genes per chromosome. These sex-specific genes could then be analyzed using cDNA arrays, hopefully yielding a manageable subset of candidate genes, the expression patterns of which could then be studied in reptilian embryos of species having either GSD or TSD.

Dosage compensation in reptiles?

Species with heteromorphic sex chromosomes are faced with the dilemma of how to achieve equal levels of gene expression between the sexes when one sex has only one copy and the other sex has two copies of a particular chromosome. Dosage compensation allows for the differential expression of sex-linked genes [16]. This phenomenon has been most intensively studied in three systems in the animal kingdom: *Drosophila*, *Caenorhabditis elegans* and mammals (primarily human and mouse). Different processes characterize each of these three systems; however, there is one essential common feature: in all three cases, specialized complexes bind to the X chromosome of one sex, modify its chromatin confirmation and regulate its transcription. Active areas of research include determination of the *cis*-acting site(s) on the X chromosome that initiate dosage compensation and elucidation of the subsequent downstream epigenetic events. Further

Figure 4

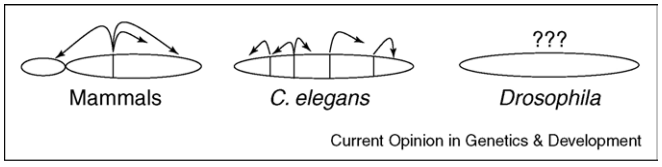


Diagram indicating the concept of 'spreading' in the process of dosage compensation in three model systems. In mammals, an important step in X chromosome inactivation is the 'coating' of the inactive X with *Xist* RNA, which is encoded by a single gene [22]. In *C. elegans*, the dosage compensation complex initially binds to multiple *cis*-acting recruitment sites on the X, and then spreads out along the entire chromosome [17]. Alternatively, spreading is not thought to occur in *Drosophila*; rather, a model involving hierarchal affinities of binding sites has been proposed [18].

studies of dosage compensation will provide greater insight into the mechanisms regulating chromatin domain organization, which is crucial toward understanding the regulation of gene expression in development [17].

In *Drosophila* males, transcription of the single X chromosome is doubled so that the amount of gene product equals that of XX females. Dosage compensation is carried out by a ribonucleoprotein complex called the dosage compensation complex (DCC) [18]. This assemblage contains six proteins: three male-specific lethals (MSL1, MSL2 and MSL3); males absent on the first (MOF); a histone acetyltransferase (HAT); and the JIL1 histone H3 kinase. In addition, *roX1* and *roX2*, two non-coding RNAs, are involved [19]. This DCC accumulates in the males' cells and coats the X chromosome. The histone H4 at lysine 16 on the X becomes acetylated, the chromatin becomes less compact and transcription is elevated. The actual mechanism of how histone modification affects transcriptional activation is unclear.

In *C. elegans*, genes on the XX chromosomes of hermaphrodites are downregulated so that their transcriptional output is equal to that of the genes in the XO male. This downregulation is initiated by a protein called SDC-2 (sex determination and dosage compensation defect-2), which assembles a collection of proteins including DPY-26 (DumPY: shorter than wild type protein-26), DPY-27, SDC-3 and MIX-1 (mitosis- and X-associated protein-1), which, collectively, are known as the DCC [20]. The DCC is recruited to multiple *cis*-acting regions of the X chromosomes of hermaphrodites and spreads out along the chromosome from these initial binding sites to effect suppression of transcription on the entire chromosome (Figure 4) [17].

In mammals, dosage compensation is achieved by the transcriptional silencing of one complete X chromosome in all cells of the female body [21]. This process of X chromosome inactivation (Xi) takes place early in development [16]. It is mediated from a single X inactivation center (*Xic*), containing a *cis*-acting gene called *Xist*, which encodes a non-coding RNA that coats the inactive X chromosome [22]. Subsequently, this process involves a series of epigenetic events, including methylation of both histones and nucleic acids, and histone hypoacetylation [23]. Furthermore, it has recently been shown that the PRC1 (protein regulator of cytokinesis 1) complex of polycomb proteins maintains the inactive state in somatic cells [24]. Finally, it is known that many human genes on the inactive X escape inactivation, and these escape genes are non-randomly distributed along the inactive X chromosome [25].

Given the extreme diversity in reptilian sex chromosome systems, one would predict that fundamentally distinct dosage-compensation systems exist in this group and that, by studying these species, novel molecular mechanisms controlling chromatin organization and gene expression in development might be discovered. In addition, we can ask whether dosage compensation occurs in polyploid reptiles such as the desert-grasslands whiptail lizard, a triploid parthenogenetic species descended from the hybrid union of two sexual species [26]; or the side-necked turtle, *Platemys platycephala*, a species having diploid–triploid somatic mosaicism (see Glossary) [27]. The study of dosage compensation has been restricted to model species because extensive genomic reagents are required for assessing gene expression of an entire chromosome. Current genomics technologies are poised to open this field to previously unstudied species. The nucleotide sequence of entire reptilian X and Z chromosomes would enable cDNA arrays and quantitative PCR to measure expression level differences between males and females [28]. Subsequent studies could determine whether novel mechanisms of gene silencing or expression are found during reptilian development.

Conclusions and future directions

Mother Nature created an excellent natural laboratory for studying the genetic control of development when she designed the sex chromosome and sex determination systems of living reptiles. Until now, progress in understanding these mysteries has been slow; however, the availability of contemporary genomics technologies such

as chromosome sorting, fluorescence *in situ* chromosome hybridization, high-throughput sequencing, subtractive hybridization and cDNA arrays are poised to bring about rapid increases in knowledge. As previous developmental genetic studies have shown, there are similarities and differences when mechanisms from various species are compared. In this vein, we can expect to learn not only reptile-specific processes but also findings that are much more general in nature. These broader, more general discoveries are of paramount interest because they will help us understand normal and abnormal development of various embryonic stages in different taxonomic groups.

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