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Chromosomal Context Affects the Molecular Evolution of Sex-linked Genes and Their Autosomal Counterparts in Turtles and Other Vertebrates

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Abstract

Sex chromosomes evolve differently from autosomes because natural selection acts distinctly on them given their reduced recombination and smaller population size. Various studies of sex-linked genes compared with *different* autosomal genes within species support these predictions. Here, we take a novel alternative approach by comparing the rate of evolution between subsets of genes that are sex-linked in selected reptiles/vertebrates and the *same genes* located in autosomes in other amniotes. We report for the first time the faster evolution of Z-linked genes in a turtle (the Chinese softshell turtle *Pelodiscus sinensis*) relative to autosomal orthologs in other taxa, including turtles with temperature-dependent sex determination (TSD). This faster rate was absent in its close relative, the spiny softshell turtle (*Apalone spinifera*), thus revealing important lineage effects, and was only surpassed by mammalian-X linked genes. In contrast, we found slower evolution of X-linked genes in the musk turtle *Staurotypus triporcatus* (XX/XY) and homologous Z-linked chicken genes. TSD lineages displayed overall faster sequence evolution than taxa with genotypic sex determination (GSD), ruling out global effects of GSD on molecular evolution beyond those by sex-linkage. Notably, results revealed a putative selective sweep around two turtle genes involved in vertebrate gonadogenesis (*Pelodiscus*-Z-linked *Nf2* and *Chrysemys*-autosomal *Tspan7*). Our observations reveal important evolutionary changes at the gene level mediated by chromosomal context in turtles despite their low overall evolutionary rate and illuminate sex chromosome evolution by empirically testing expectations from theoretical models. Genome-wide analyses are warranted to test the generality and prevalence of the observed patterns.

Subject area: Bioinformatics and computational genetics, Molecular adaptation and selection
Key words: Amniote vertebrates, Faster-X and Faster-Z effect, Molecular evolution of coding DNA sequences, Sex chromosome evolution, Temperature-dependent and genotypic sex determination, TSD and GSD reptiles

Sex chromosomes evolved repeatedly and independently from autosomes in multiple branches of the tree of life (Charlesworth 1991; Bachtrog et al. 2014). Animal sex chromosomes are often heteromorphic (Tree of Sex Consortium et al. 2014), but may be homomorphic as in some snakes and ratite birds (Ogawa et al. 1998; Ellegren 2000).

A longstanding question in evolutionary biology is the extent to which sex chromosomes and autosomes differ in their molecular evolution (Vicoso and Charlesworth 2006) given that in species with heteromorphic sex chromosomes, heterogametic individuals (e.g. XY males or ZW females) are hemyzygous for sex-linked genes present in the X or Z but absent in the Y or W chromosome. For instance, selection acting on partially or fully recessive alleles in hemizygous state, under reduced recombination, can facilitate the accumulation of beneficial mutations and the removal of deleterious ones faster on sex chromosomes than autosomes (Charlesworth et al. 1987; Mank et al. 2007). Further, all other things being equal, population size is lower for sex chromosomes than autosomes (three-fourths for X and Z, and one-fourth for Y and W compared to autosomes) (Bachtrog et al. 2011), such that genetic drift may be relatively stronger than natural selection in sex chromosomes, thus facilitating the fixation or loss of alleles despite their fitness effects (Bachtrog et al. 2011). Mating system can also play an important role on the molecular evolution of sex chromosomes due to its influence on effective population sizes, and this effect also differs between X and Z chromosomes (Vicoso and Charlesworth 2009; Mank et al. 2010b; Bachtrog et al. 2011). These faster-X/faster-Z hypotheses have been supported in diverse taxa (e.g. *Drosophila* [Mackay et al. 2012; Campos et al. 2014;], moths [Sackton et al. 2014], birds [Mank et al. 2007], mice [Kousathanas et al. 2014], humans and chimpanzees [Lu and Wu 2005]). While invaluable, these studies examined exclusively closely related species that shared a common sex chromosome system by comparing the molecular evolution of sex-linked genes versus *different* autosomal genes in the same species. Instead, here we take a phylogenetically broader approach, leveraging the diversity of sex-determining mechanisms found in reptiles (particularly turtles), to test whether the rate of evolution of sex-linked genes in reptiles and select vertebrates with sex chromosomes differs from that of the *same* genes located in autosomes in other amniotes. Turtles and squamates are ideally suited to address these issues because they possess both male and female heterogametic systems of genotypic sex determination (GSD: XX/XY and ZZ/ZW), plus temperature-dependent sex determination (TSD) without sex chromosomes that serve as a negative control (Valenzuela and Lance 2004; Tree of Sex Consortium et al. 2014; Valenzuela et al. 2014). Yet the relative rate of evolution of DNA sequences between sex chromosome and autosomes is unexplored in reptiles, partly because the content of reptilian sex chromosomes remains understudied and our knowledge is thus fragmentary (Kawagoshi et al. 2009; Kawagoshi et al. 2012; Kawagoshi et al. 2014; Montiel et al. 2016; Montiel et al. 2017).

As a first step to fill this gap, here we conducted comparative analyses of the rates of non-synonymous to synonymous mutations (dN/dS = ω) of coding sequences from 6 turtles (with XX/XY, ZZ/ZW, and TSD), an anole (XX/XY), the alligator (TSD), chicken (ZZ/ZW) and 2 mammals (XX/XY). With these data we tested the hypothesis that the rate of evolution (ω) differs for sequences in a sex-chromosomal context (ω_{SL}) versus an autosomal context (ω_{A}) using a likelihood ratio test that fits a common rate for all the data (null model) and compares it to an alternative model that fits separate rates for sex-linked versus autosomal genes. We contrasted these results with the results of the same analysis performed on control genes that are not sex-linked (Figure 1). In a secondary analysis, we assessed whether selection drives the molecular evolution of these sequences and identified the sites that are targeted using a branch-site model. We observed for the first time faster evolution driven by positive selection of Z-linked sequences in some turtles and slower evolution of X-linked sequences in others. We also tested whether molecular rates differ in reptiles compared to other amniotes, and between TSD and GSD vertebrates, to examine potential lineage effects among taxonomic groups, or the effect of sex-determining mechanism on molecular evolution. It might be expected that if GSD *per se* affects molecular evolution beyond the effect of sex-linkage, then orthologous sequences of sex-linked sequences should exhibit lower rates of evolution in TSD taxa. Finally, because we detected exceptional rates of evolution in two genes in a GSD and a TSD turtle, we explored these genes in more detail, identified mutations in their 3D protein structure that may alter their function, and uncovered a potential selective sweep through a genomic region harboring gene regulators of sexual development. .

Methods

Data Collection and Processing

Sequence data were obtained from NCBI (Acland et al. 2014) genome assemblies for the Chinese softshell turtle *Pelodiscus sinensis* (PSI) (Wang et al. 2013), painted turtle *Chrysemys picta* (CPI) (Shaffer et al. 2013), American alligator *Alligator mississippiensis* (AMI) (St John et al. 2012), anole lizard *Anolis carolinensis* (ACA) (Alföldi et al. 2011), chicken *Gallus gallus* (GGA) (Hillier et al. 2004), human *Homo sapiens* (HSA) (Venter et al. 2001), and mouse *Mus musculus* (MMU) (Waterston et al. 2002) as described below (assemblies PelSin_1.0, ChrPicBel3.0.1, AKHW00000000.1, AnoCar2.0, Gallus_gallus-5.0, GRCh38.p3, and GRCm38.p4, respectively). Sequence data from the spiny softshell turtle *Apalone spinifera* (ASP), musk turtle *Staurotypus triporcatus* (STR), wood turtle *Glyptemys insculpta* (GIN), and Murray river turtle *Emydura macquarii* (EMA) were obtained from next-generation genome sequencing as part of other projects (Valenzuela et al., unpublished data). These data consisted of short-read paired-end Hi-Seq Illumina sequencing of one male and one female per species, one lane per individual. Hereafter, species will be referred to by their genus names or 3-letter acronyms.

Sequences of sex-linked genes from human, mouse, chicken, anole, and *Apalone* turtle were used as probes to detect their autosomal gene orthologs in the remaining species wherever present using GMAP (Wu and Watanabe 2005). Complete coding sequences were extracted from the Ensembl genome browser (www.ensembl.org) for 4 genes from each set (Figure 1). For human, mouse, chicken, and anole, 20 genes that are sex-linked in our focal species and autosomal in others, plus 4 control genes, were chosen at random from a uniform distribution (Figure 1). Chicken-specific transcripts were mapped against available genomes of the *Pelodiscus* turtle, *Chrysemys* turtle, and *Alligator*, and against newly sequenced lower-coverage genomes we obtained for *Staurotypus*, *Apalone*, *Glyptemys*, and *Emydura* turtles from another project using discontiguous megablast in Geneious version R7.1.7, applying default parameters (Kearse et al. 2012). Novel sequence data used in this study were deposited in GenBank (accession numbers MF686705-MF686800, detailed in Supplementary Table S3) and their alignments can be found in the Supplementary Information. For each top BLAST hit, the coding sequence from the corresponding gene was annotated in the newly sequenced turtle genomes (*Staurotypus*, *Apalone*, *Glyptemys*, and *Emydura*) after ensuring that the alignments were in frame, and extracted. Stop codons from the ends of coding sequences were

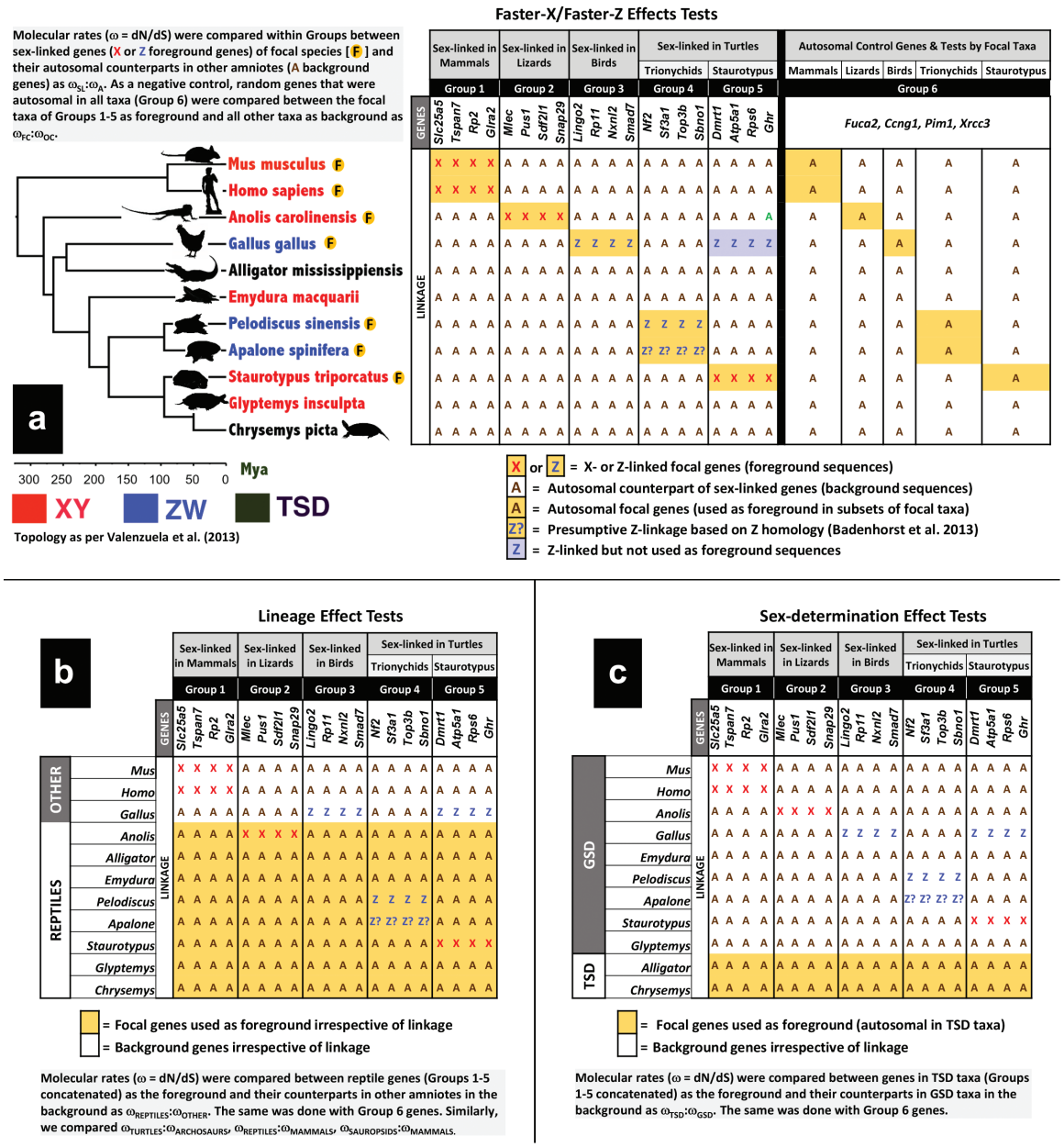

Figure 1. **(a)** Phylogenetic relationships among selected vertebrates, their sex-determining mechanism, and focal genes of interest whose molecular evolution was examined in this study. Sex-linked genes were chosen from Human (*Homo sapiens*), Mouse (*Mus musculus*), Chicken (*Gallus gallus*), the musk turtle (*Staurotypus triporcatus*), the Trionychid softshell turtles (*Apalone spinifera, Pelodiscus sinensis*) and the Anole lizard (*Anolis carolinensis*). Comparisons were carried out within each gene group between sex-linked genes of interest in focal taxa (yellow cells) and their autosomal counterparts (white 'A' cells) in other amniotes. Control genes we chosen at random from autosomal non-housekeeping genes and were compared between the same focal taxa from groups 1–5 and the other amniotes. **(b)** Comparison of reptiles versus other amniotes was carried out for the 20 genes in groups 1–5 concatenated. **(c)** Comparison of TSD versus GSD vertebrates was carried out for the 20 genes in groups 1–5 concatenated, and for each gene group 1–5 separately (Supplementary Table S1). See online version for full colors.

removed from all coding sequences. The content of the sex chromosomes of *Glyptemys* or *Emydura* was unknown, such that only autosomal sequences from these species were used to maximized the turtle dataset (they provided additional background autosomal sequences in the analyses of sex-linkage effects as described below). Coding sequences were concatenated, and a multiple-sequence translational alignment was performed using Geneious version R7.1.7 (Kearse et al. 2012). All multiple sequence alignments were visually inspected prior to further analysis to ensure they had in frame codons and no premature stop codons (none were found). Conserved blocks from the multiple sequence alignments were identified for further analysis using Gblocks (Castresana 2000), after visually inspecting for possible misalignment at gene junctions. All subsequent analyses were performed in a single step on this concatenated alignment of coding sequences to obtain an overall assessment of the rate of evolution for each gene group (Groups 1–5, Figure 1), given that our objective was to assess the general effect of sex chromosome linkage on molecular evolution (an overall faster-X/faster-Z) rather than locus by locus effects. Specifically, these data were used in various molecular evolutionary analyses comparing the ratio of synonymous to synonymous substitutions among groups as described below to 1) test if sex-linked sequences and their autosomal counterparts evolved at different rates,

2) to test for major lineage effects by testing if reptilian and sauropsid (reptiles + birds) sequences evolved at a different rate than mammals, or if archosaurs (birds + crocodilians) sequences evolved at different rates than turtles, and 3) to test if sequences in TSD taxa evolved at a different rate than in GSD taxa (which inform whether GSD and not just sex chromosome linkage might influence molecular evolution). All sex-linked genes in a focal species had autosomal orthologs in all other taxa, except for genes in PSI-Z which were also sex-linked in the homologous ASP-Z (Badenhorst et al. 2013), and genes in STR-X which is homologous to GGA-Z (Kawagoshi et al. 2014). To serve as negative controls, we randomly chose autosomal genes lacking housekeeping functions and which therefore, should be under no special selective constraint. For this purpose, we subtracted a list of housekeeping genes (Eisenberg and Levanon 2013) (http://www.tau.ac.il/~elieis/HKG/HK_genes.txt) from the set of all autosomal human genes, and randomly selected four genes, extracted their coding sequences from all taxa, concatenated them and multiple-aligned them as described above. The size of the raw concatenated multiple coding sequence alignments obtained ranged from 3.6 kb (for genes that are sex-linked in *Anolis* and autosomal in others) to 11 kb (for genes that are sex-linked genes in *Apalone*/*Pelodiscus* and autosomal in others), and 3.98 kb for the control genes. After filtering the alignments for conserved blocks (using Gblocks) and non-gapped regions (using PAML), the final alignments ranged from 2.4 kb (*Anolis*) to 8.5 kb (*Apalone*/*Pelodiscus*) for the sex-linked genes, and 3.61 kb for the control autosomal genes.

Model Testing of Molecular Evolution

The CODEML package in PAML version 4.7 (Yang 2007) was used for all molecular evolution analyses. The rate of coding sequence evolution was measured as the ratio of non-synonymous (NS) to synonymous substitutions (ω = dN/dS). To test if molecular rate of evolution (ω) was significantly different in a given sex chromosome "foreground" (ω_{SL}) relative to the autosomal "background" (ω_A), we used a likelihood test (Yang and Nielsen 1998). This approach fits a "two-ratio model" assuming ω_{SL} differs from ω_A (i.e., ω_{SL}: $\omega_A \neq 1$) and compares it to the null "one-ratio" model which assumes ω_{SL} equals ω_A (ω_{SL}: $\omega_A = 1$) and fits a single rate for both groups. Specifically, the one-ratio model is a branch model (Yang and Nielsen 1998) with the following parameters: Model = 0, NS-sites = 0; where ω *is invariant* across branches. The alternative two-ratio model uses Model = 2, NS-sites = 0; where ω is estimated from the alignment and *varies* across branches. The better-fit model was selected by computing the test statistic as: $2(\text{log-likelihood}_{\text{two-ratio}} - \text{log-likelihood}_{\text{one-ratio}})$, and using a chi-square test of significance with one degree of freedom to obtain the *P* value. A *P* value < 0.05 was used to reject the null one-ratio model and support the 2-ratio model (Supplementary Table S1). This approach was implemented for sex-linked sequences (ω_{SL}) and their autosomal counterparts (ω_A) for each subset of genes of interest by focal taxa (gene Groups 1–5 in Figure 1), as well as for the control genes (ω_C) (Group 6 in Figure 1) where the control genes from each group of focal taxa (from Groups 1–5 analyzed separately) were used as foreground and sequences from other species as background (ω_{FC}: ω_{OC}) (Figure 1). The branch model test was also used to compare the rates of evolution between TSD and GSD taxa (ω_{TSD}: ω_{GSD}) separately for genes in Groups 1–5 (Supplementary Table S2), as well as using the 20 genes of interest concatenated (Groups 1–5 concatenated) (Figure 1), to investigate whether GSD might affect the rate of molecular evolution more globally than just in the sex chromosomes. Additionally, we compared the rate of molecular evolution among various taxonomic groups ($\omega_{REPTILE}$, ω_{MAMMAL}, $\omega_{SAUROPSID}$, $\omega_{ARCHOSAUR}$) to test for major lineage effects.

Tests of selection

To better understand the molecular evolution of the genes of interest, we investigated whether the sequences examined above evolved under selection and what sites might be targeted. Under positive selection ω is expected to be >1 (higher rate of non-synonymous than of synonymous mutations), while under purifying selection ω is expected to be <1. A ω equal to 1 implies that there is no difference between the rate of non-synonymous and synonymous mutations, and thus it is a signature of neutral evolution. To generate confidence intervals for ω, 100 bootstrapped alignments were generated by sampling codons with replacement (Table 1).

To test whether specific sites were undergoing positive selection, the branch-site model (Yang et al. 2005) (Model = 2, NS-sites = 2), was used to test the alternative hypotheses that (H1) ω is variable versus the null hypothesis (H0) that ω is invariant, using the following PAML parameters: For H0, omega = 1 (initial guess), fix omega = 1 (ω not allowed to vary). For H1, omega = 1 (initial guess), fix omega = 0 (estimates optimal ω for the branches). The Bayes empirical Bayes (BEB) score ($P > 0.9$) was used to identify sites under positive selection (Yang et al. 2005). These analyses were repeated separately for the control genes. The following phylogenetic tree was used in all molecular evolution-based analysis: ((MMU, HSA), (ACA, ((GGA, AMI), (EMA, ((PSI, ASP), (STR, (GIN, CPI)))))))) (Valenzuela et al. 2013) (Figure 1). Because the analyses described above detected remarkably high rates of evolution of *Nf2* in *Pelodiscus* (see Results), separate branch- and branch-site model analyses were also performed for the expanded gene block neighboring *Nf2*, which included *Zmat5*, *Cabp7*, *Nipsnap1*, and *Ccdc157*. However, homologs of these genes could not be adequately annotated in *Anolis*, *Staurotypus* and *Emydura* due to absence of data/missing exons in the available genomes. Thus, *Anolis*, *Staurotypus* and *Emydura* were excluded from the analysis of the gene block neighboring *Nf2* and the phylogenetic tree was pruned accordingly.

Protein Sequence and structure analysis

De novo 3D structures for protein sequences which exhibited particularly high rate of molecular evolution in the above analyses (for NF2 in *Pelodiscus*; and TSPAN7 in *Chrysemys*; see Results below) were predicted using the i-Tasser web server (Zhang 2008) and compared to the 3D structures in their closest relatives in the dataset (*Apalone* and *Glyptemys*, respectively) to identify protein regions of potential functional importance affected by these mutations. Pymol (Schrödinger 2010) was used for structural alignment of protein structures. Domain identification on the NF2 and TSPAN7 protein sequences was performed using the BindN (Wang and Brown 2006) and the PROSITE web servers (Sigrist et al. 2013).

Results

Is Molecular Evolution Faster in a Sex-Chromosomal Context Versus an Autosomal Context?

Here we compared the rate evolution (ω = dN/dS) between coding sequences that are sex-linked in focal mammals, lizard, chicken, and turtles to their autosomal orthologs in other taxa to test for the effect of sex-chromosome evolution on the molecular evolution of the genes they harbor. We detected significant evolutionary patterns

Table 1. Faster-X index computed from the branch model

	Sex-linked genes in focal taxa						Control genes	
Gene Group	Group 1	Group 2	Group 3	Group 4		Group 5	Group 6	
Focal Taxa	HSA/MMU	ACA	GGA	ASP	PSI	STR	All but GGA	GGA
Linkage	X	X	Z	Z	Z	X	Autosomal	Autosomal
Original alignment length (bp)	4161	3636	4788	11004	11004	5697	4820	4820
GBlocks alignment (bp)	3777	3042	4242	10821	10821	4668	3975	3975
PAML alignment (bp)	3402	2385	3396	8451	8451	3333	3612	3612
Average ω_{SL} (sex-linked sequences)	0.4608	0.0892	0.0389	0.0412	0.1363	0.0533	0.1333	0.0868
ω_{SL} 95% CI (left)	0.2363	0.0877	0.0371	0.0407	0.133	0.0513	0.1319	0.0846
ω_{SL} 95% CI (right)	0.6853	0.0907	0.0407	0.0417	0.1396	0.0553	0.1348	0.089
Average ω_A (autosomal background)	0.0803	0.0892	0.0636	0.0412	0.0362	0.0986	0.1333	0.1418
ω_A 95% CI (left)	0.079	0.0877	0.0625	0.0407	0.0357	0.0972	0.1319	0.1403
ω_A 95% CI (right)	0.0816	0.0907	0.0647	0.0417	0.0367	0.1	0.1348	0.1433
Better-fit model (P value of likelihood ratio test)	Two-ratio $P < 0.0001$	One-ratio $P = 0.07$	Two-ratio $P = 0.02$	One-ratio $P = 0.51$	Two-ratio $P < 0.0001$	Two-ratio $P < 0.0001$	One-ratio All $P > 0.12$	Two-ratio $P = 0.0001$
Relative rate of evolution (ω_{SL}: ω_A or ω_{FC}: ω_{OC})	5.738	1	0.6116	1	3.765	0.5406	1	0.612

Gene groups as per Figure 1. Likelihoods were estimated for the one-ratio and two-ratio branch models using CODEML, and the better-fit model was chosen using a likelihood ratio test (chi-square test with one degree of freedom). The ω values presented correspond to the best-fit model. The ω_{SL}: ω_A or ω_{FC}: ω_{OC} ratio equals 1 when the null model (one-ratio model) is the best-fit model. The best-fit model for control genes (Group 6 in Figure 1) was the one-ratio model which had identical ω values in all taxa except GGA (ASP/ PSI, STR, HAS/MMU and ACA). For GGA, the 2-ratio model provided best-fit to the data. A = Autosomal, ACA = *Anolis*, ASP = *Apalone*, C = control genes, CI = confidence interval, FC = Foreground control genes, GGA = *Gallus*, HSA= *Homo*, MMU = *Mus*, OC = Other control genes, PSI = *Pelodiscus*, SL = Sex-linked, STR = *Staurotypus*, ω = dN/dS ratio.

in some genes and taxa that are attributable to their chromosomal context, sometimes concordant with a faster-X/Z pattern, but sometimes not. For instance, the relative rate of evolution (ω_{SL}: ω_A) measured on bootstrapped alignments using the branch model for human/mouse was greater than 1 (ω_{SL}: ω_A = 5.74, $P < 0.0001$, Table 1), indicating that these X-linked sequences in mammals (Group 1 Figure 1) undergo faster evolution than the same sequences in an autosomal context in other vertebrates, and rates were similar for human and mouse (χ^2: 0.04; *P* value: 0.84)]. The opposite was true in chicken whose Z-linked sequences (Group 2 Figure 1) evolve slower than their autosomal counterparts in other taxa (ω_{SL}: ω_A = 0.61, $P = 0.0001$) (Figure 2A). Among reptiles, X-linked genes in *Anolis* (Group 3 Figure 1) evolve at the same rate than their autosomal counterparts (ω_{SL}: ω_A = 1, $P = 0.07$), but contrasting patterns were detected among turtles. Namely, Z-linked genes in *Pelodiscus* (Group 4 Figure 1) evolve faster than their autosomal orthologs (χ^2: 101.66; $p < 0.0001$), whereas the same Z-linked genes in its close relative *Apalone* appear to evolve at the same rate than their autosomal counterparts (ω_{SL}: ω_A = 1, $P = 0.51$) (Table 1). In contrast, X-linked genes in *Staurotypus* (Group 5 Figure 1) evolve slower than the same sequences in autosomes (χ^2: 13.88; $P < 0.0001$). Table 1 documents the rates of evolution (ω) for each lineage, along with the relative rate of evolution (ω_{SL}: ω_A) for the best-fit model. Supplementary Table S1 contains the complete list of branch model comparisons. Because the XY of *Staurotypus* and ZW of chicken are homologous and arose from the same ancestral pair of chromosomes (Kawagoshi et al. 2014), we also tested the evolution of their sex-linked genes combining these 2 taxa as "focal species" with the remaining taxa in the background, and did not observe a significantly different rate of evolution of their sex-linked sequences relative to the autosomal orthologs in the remaining vertebrates [χ^2 = 0.018, $P = 0.89$], indicating different evolutionary trajectories between *Staurotypus* and chicken that mask each other when combined. Similarly, we combined the 2 Trionychid turtles in our dataset (*Apalone* and *Pelodicus*) as focal species and found no difference in the rate of evolution of their sex-linked genes relative to the autosomal orthologs in the other taxa (χ^2 = 0.46; *P* value: 0.49), indicating that the relatively slower evolution in *Apalone* obliterates the faster pattern observed in *Pelodiscus* alone. To test whether the patterns observed for sex-linked genes in any taxa were due to their sex-chromosomal context or solely to lineage-specific effects, we carried out the same comparisons using the random set of control genes (Group 6 Figure 1), and found no difference in ω between foreground (focal species) and background (other taxa) values in any species except chicken, whose control genes evolved significantly slower than in other amniotes (Table 1, Figure 2b).

Is Molecular Evolution Affected by Sex Determination Beyond Sex-Linkage?

To test whether GSD *per se* and not just sex-linkage might influence molecular evolution, we compared the rates between TSD and GSD taxa and found faster molecular evolution in TSD than in GSD vertebrates for some genes and not others. In particular, the autosomal homologs of the 4 mammalian sex-linked genes evolved much faster in *Chrysemys* and *Alligator* (both TSD), relative to the GSD background (Supplementary Table S2), whereas no other group of TSD-homologs of sex-linked genes from other GSD taxa did. Furthermore, results using the branch model on

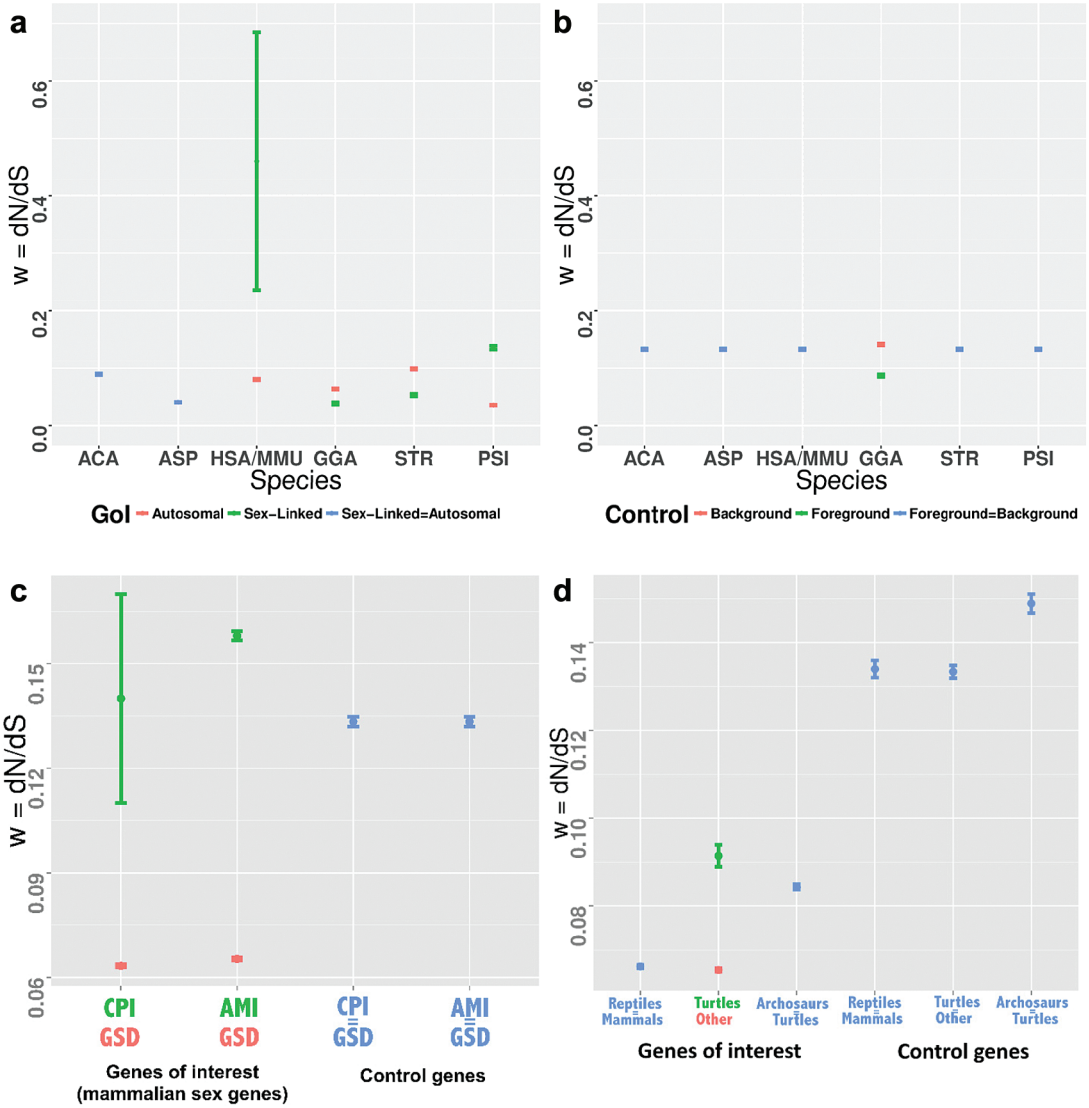

Figure 2. (**a**) Molecular evolution [dN/dS (ω) ratio] of sex-linked (green) and autosomal sequences (red) measured in selected vertebrates as determined by the branch model. Analyses used the sex-linked sequence in each focal species (x-axis) as foreground and the autosomal orthologs from every other species as background. Blue ω values denote cases where the green and red dots overlap (i.e. no differences were detected between groups). (**b**) Molecular evolution measured by an identical analysis of control genes from each focal species (green), background species (red). (**c**) Molecular evolution in the genes of interest (mammalian sex-linked genes) and control genes measured in focal vertebrate TSD species (CPI or AMI; green) against GSD species (red). Error bars represent 95% confidence interval ranges from bootstrapped alignments among all foreground sequences and all background sequences in each comparison, respectively. ACA: *Anolis carolinensis*; ASP: *Apalone spinifera;* HSA: *Homo sapiens*; MMU: *Mus musculus*; GGA: *Gallus gallus*; STR: *Staurotypus triporcatus*; PSI: *Pelodiscus sinensis*; CPI = *Chrysemys picta*; AMI = *Alligator mississippiensis*. (**d**) Rates of molecular evolution [ω = dN/dS] in the genes of interest and control genes measured in focal clades (green) against background clades (red). GoI: genes of interest (groups 1-5 in Figure 1). Control: control genes (group 6 in Figure 1). See online version for full colors.

the concatenated alignment of all 20 genes across 11 species revealed that the painted turtle *Chrysemys* exhibits a higher rate of evolution relative to all other GSD vertebrates (χ^2: 86.26; $P < 0.0001$). The same result held for *Chrysemys* when the alligator was included in the background (χ^2: 100.04; $P < 0.0001$), or for *Alligator* (albeit less strongly) with *Chrysemys* in the background ($\chi^2 = 4.47$; P value = 0.03, or without *Chrysemys* in the background: $\chi^2 = 7.81$; $P = 0.005$) (Figure 2c). In contrast, rates for negative control genes from *Alligator* and *Chrysemys* were similar to their rates in GSD taxa, and resemble the rate in TSD species for genes that were sex-linked in GSD species (Figure 2c). Thus, if anything, GSD appears to decelerate the molecular evolution of some genes compared to TSD.

Are There Lineage Effects That Influence Molecular Evolution?

Comparison among vertebrate groups of all the genes in our concatenated dataset that are sex-linked in any of our focal species (gene Groups 1–5 from Figure 1 combined), revealed faster overall evolution in turtles than any other taxa (χ^2: 6.25; P value: 0.01, Figure 2d). However, this pattern disappears when separate analyses were conducted on each group of turtle homologs of the

sex-linked genes from each focal GSD taxa (gene groups 1,3,4,5 in Figure 1 analyzed individually), except for the homologs of chicken-Z genes (gene group 2 in Figure 1) which exhibited a more pronounced accelerated evolution in turtles (χ^2: 8.82; *P* value = 0.0029) (Supplementary Table S2). On the other hand, no rate differences were detected in the full set of genes (gene groups 1–5 in Figure 1 combined) between reptiles and mammals (χ^2: −0.002; *P* value: 1), between archosaurs (birds + crocodilians) and turtles (χ^2: 0.016; *P* value: 0.89), or between sauropsids (reptiles + birds) and mammals (χ^2: 0.002; *P* value: 1) (Supplementary Table S1). In contrast, group level comparisons showed that negative control genes (gene group 6 in Figure 1), which were autosomal in all taxa, evolved at a higher rate than sex-linked genes in all of our study species, but this rate was identical in all comparisons between focal species relative to the background (Figure 2d). Thus, lineage effects on molecular evolution exist but they are gene-specific and not generalizable.

Does Selection Drive the Evolution of Sex-Linked Genes in GSD Turtles or Their Autosomal Orthologs in TSD Turtles?

Overall, the branch-site model detected purifying selection in 93% of the sites in all genes, consistent with the coding nature of the sequences in the dataset. The ratio of rates between any 2 groups examined here permits assessing whether this overall purifying selection acting on these coding sequences is accentuated, equal or relaxed in one group relative to the other (e.g. ω_{SL}: ω_A). For instance, the faster-Z evolution in *Pelodiscus* suggests that these genes are under relatively relaxed purifying selection or relatively positive selection compared to their autosomal counterparts (ω_{SL}: ω_A = 3.76, $p < 0.0001$), whereas no differences were observed for the same Z-linked genes in *Apalone* (ω_{SL}: ω_A = 1, *P* = 0.51) (Table 2). In contrast, the slow-X evolution in *Staurotypus* suggests that these genes are under relatively stronger purifying selection than their autosomal orthologs (ω_{SL}: ω_A = 0.54, $P < 0.0001$).

Given the accelerated evolution of sex-linked genes in turtles observed here (Figure 2d), we explored the genes that might be the target of selection. The branch-site model identified multiple amino acid residues undergoing positive selection in the X-linked *Atp5a1* gene in *Staurotypus*, and the Z-linked *Nf2* and *Sf3a1* genes in *Pelodiscus*, relative to autosomal sequences in the remaining species. Most positively selected residues were concentrated in the *Nf2* gene in *Pelodiscus* (Supplementary Figure S1). To test whether selection was restricted to these genes only or targeted nearby genes also, we ran the branch model and branch-site model analyses on the region surrounding *Nf2* in *Pelodiscus* and detected faster evolution of a sex-linked block containing *Zmat5*, *Cabp7*, *Nipsnap1* and *Ccdc157* (dN/dS = 0.20) relative to their autosomal counterparts (dN/dS = 0.08), and encompassing 130 positively selected residues (mostly in *Nf2*). Also notably, the branch-site model predicted 39 positively selected residues (BEB score > 0.9) in the *Tspan7* gene in *Chrysemys* whereas no other gene exhibited a selection signature in this TSD turtle.

Table 2. Percentage similarity between Z-chromosomal coding sequences in PSI and ASP

PSI Scaffold	Gene	% Similarity
JH212668.1	*Mtmr3*	95.5
JH211589.1	*Ascc2*	94.1
JH211589.1	***Zmat5***	***22.8***
JH209995.1	***Cabp7***	***87.6***
JH209995.1	***Nf2***	***71.2***
JH209995.1	***Nipsnap1***	***73.5***
JH209995.1	***Ccdc157***	***50.0***
JH209995.1	***Sf3a1***	***79.1***
JH204957.1	*Top3b*	98.7
JH204957.1	*Sdf2l1*	95.7
JH204957.1	*Ydjc*	93.6
JH204957.1	*Ubel2l3*	93.1

Stronger differences (i.e. similarities < 90%) are highlighted in bold. % Similarity = (Query coverage * % Identity).

Are Sites Under Selection in Turtles Important for Protein Structure?

Given the exceptional rates of evolution of *Nf2* in *Pelodiscus* and of *Tspan7* in *Chrysemys*, we examined further the predicted 3D structure of the NF2 and TSPAN7 proteins to test if changes occurred at functionally important sites. Our *de novo* predictions for NF2 revealed differences in root-mean-square deviation (rmsd) in the alpha-helices (Figure 3a) towards the C-terminal end of the protein between the softshell turtles *Pelodiscus* and *Apalone* (Trionychidae), with most positively selected residues at the FERM domain which helps localize proteins to the plasma membrane (Pearson et al. 2000) (Supplementary Figure S1). A much greater structural difference was predicted for TSPAN7 between *Chrysemys* and *Glyptemys* (Emydidae) (Figure 3b), although only 2 out of 39 positively selected residues that differ between these 2 turtles are located in the transmembrane family domain (Supplementary Figure S2).

Discussion

Sex chromosomes are predicted to experience a distinct tempo and mode of evolution compared to autosomes, such as faster mutation accumulation via selection of recessive beneficial mutations (Charlesworth et al. 1987; Mank et al. 2007), or the loss or fixation of mutations via drift (Bachtrog et al. 2011; Mank et al. 2010a). Alternatively, sex chromosomes may evolve slower when selection acts on standing rather than novel mutation (Orr and Betancourt 2001). This context-dependent evolution has been examined in some vertebrates and some invertebrates (Betancourt et al. 2002; Lu and Wu 2005; Mank et al. 2007; Hu et al. 2013; Avila et al. 2014; Sackton et al. 2014) but never in turtles or other reptiles. As a first step to fill this gap, here we take a novel and complementary approach from previous studies that compared the molecular evolution of sex-linked genes versus different autosomal genes in the same species. Instead, we leverage the diverse sex-determining mechanisms of reptiles to test whether the rate of evolution of a small set of sex-linked genes in reptiles and select vertebrates with XX/XY or ZZ/ZW chromosomes differs from that of the same genes located in autosomes in other amniotes, with particular emphasis on turtles.

Overall, we found that as expected, sex-linkage affects molecular evolution compared to an autosomal context, but the pattern is not uniform for all gene groups, types of heterogamety, or taxonomic group. We also detected differences in the rates of evolution between sex-determining systems (TSD vs GSD) which counter the notion that GSD might accelerate molecular evolution overall as is predicted for sex-linkage under the faster-X/Z hypothesis. Interestingly, turtles displayed faster evolution of sex-linked genes and of some of their autosomal orthologs, which appears driven by selection, and in two remarkable cases examined further, the implicated sites may alter protein function. Below we describe these findings in more detail.

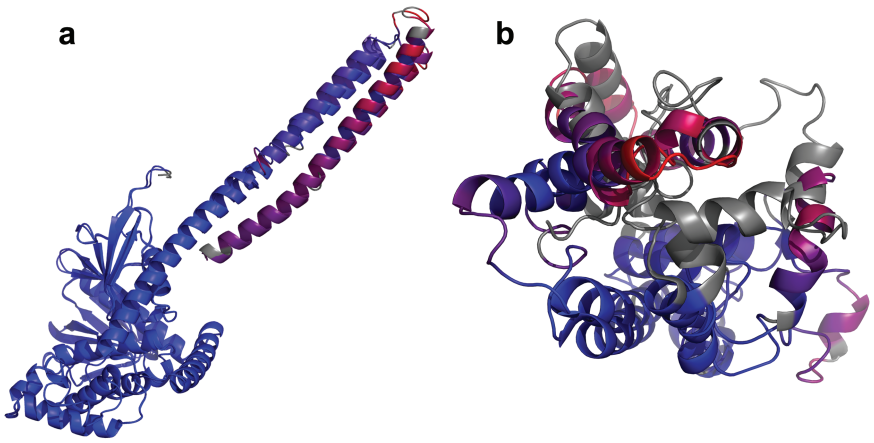

Figure 3. Overlaid structural alignment of (**a**) NF2 proteins in *Pelodiscus sinensis* and *Apalone spinifera* (**b**) Tspan7 proteins in Chrysemys picta and *Glyptemys insculpta*. Structures were computed using the i-Tasser web server. The rmsd between structures is indicated by a spectrum of colors, with higher root-mean-square deviation (rmsd) indicated in red, lower rmsd in blue. Regions that could not be aligned are indicated in grey. See online version for full colors.

Faster-X and Slower-Z Genes in Mammals and Birds

Sex-linked genes in mammals (*Homo/Mus*) evolved at an accelerated rate relative to their autosomal orthologs in other species (ω_{SL}: $\omega_A > 1$, $P < 0.0001$, Table 1), indicating that in general, mammalian X-genes accumulate disproportionately more changes at the amino acid level that could potentially alter protein function when compared to birds and reptiles (no differences existed between human and mouse). This faster-X pattern is concordant with previous observations between reptiles and mammals (Janes et al. 2010; Shedlock et al. 2007). In contrast, we observed for the first time that *Anolis* X-linked genes evolve at a similar rate than the same sequences in an autosomal context in other amniotes (ω_{SL}: $\omega_A = 1$, $P = 0.07$, Table 1). Interestingly, chicken-Z linked genes evolved slower than their autosomal orthologs, contrasting with the faster-Z evolution reported earlier in birds (Wright et al. 2015). This discrepancy may be explained by the different approaches between studies. Namely, previous studies compared sex-linked sequences against *different* autosomal sequences in the same species, such that results could not disentangle gene-specific effects from chromosome-specific effects. In contrast, our approach compares the evolution of the *same genes* in different chromosomal contexts (i.e. sex-linked versus autosomal) across various amniote groups, allowing us to decouple gene-specific effects from the chromosomal context effects, and to identify lineage effects as well. We speculate that the observed differences between mammalian-X and chicken-Z might happen because mammalian-X contains few direct regulators of gonadogenesis (Karkanaki et al. 2007), whereas chicken-Z carries *the* master sex-determining gene which acts via gene dosage (Churchill and Storey 1991) such that selection may be stronger to prevent disruption of avian gonadal determination/differentiation in chicken-Z than in mammalian-X. Further research is needed to test this hypothesis directly. Effective population size (N_e) may influence sex chromosome evolution relative to autosomes (Vicoso and Charlesworth 2009; Mank et al. 2010b) such that at very large N_e (e.g. *Drosophila*) selection against deleterious mutations (which dampens the faster-X/Z effect) may prevail over selection for recessive beneficial mutations (which accelerate the faster-X/Z effect) (Mank et al. 2010b). Perhaps differences in N_e account for some of our findings because birds have much larger N_e compared to human and mouse (Mank et al. 2010b), yet these two mammals displayed fairly identical patterns despite their substantially different N_e. The effect of mating system in our findings is also unclear (Vicoso and Charlesworth 2009; Mank et al. 2010b; Bachtrog et al. 2011) because polygyny (via male–male competition or female-choice) reduces N_{eZ} and increases N_{eX} from their three-fourths N_{eA} under monogamy (Bachtrog et al. 2011), facilitating drift in the Z and selection on the X, yet here we observed faster-X in mammals and slower-Z in birds. Dosage compensation is another factor reported to affect sex-chromosome evolution such that in its absence, the faster-X effect should slow down for beneficial mutation and accelerate for deleterious mutations (Charlesworth et al. 1987) as may happen for faster-Z in birds (Mank et al. 2010b), yet here we detected faster-X and slower-Z.

Slower-X in *Staurotypus* Turtles Matches Homologous Slower-Z in Chicken

Staurotypus-X genes evolved slower than their autosomal counterparts, suggesting the intensification of purifying selection, or lack of recent mutations, since theory predicts that selection acting on standing genetic variation rather than on novel mutations can decelerate sex chromosomes evolution compared to autosomes (Orr and Betancourt 2001). The relatively young age of *Staurotypus* XX/XY system (~50 million years) compared to the ~180 million years old ZZ/ZW in *Pelodiscus* and *Apalone* (Badenhorst et al. 2013) supports the lack of novel mutations hypothesis, given the overall slow rate of molecular evolution in turtles (Shaffer et al. 2013). Alternatively, perhaps the genes examined here are located in the pseudoautosomal region (PAR) of STR-X where recombination with the Y prevails, thus weakening genetic drift and making this region behave as an autosome (Otto et al. 2011). Further, because no Y-specific genes in *Staurotypus* are known, analyses of sex-linked genes that do not undergo homologous recombination is precluded. The slower evolution of *Staurotypus*-X and chicken-Z genes is particularly interesting, because both arose from the same ancestral autosome pair (Kawagoshi et al. 2014). Whether the pattern for these four genes in chicken and *Staurotypus* is an ancestral condition, or arose independently *after* sex chromosome evolution in each lineage remains to be seen. Also unknown is whether *Staurotypus* sex determination relies on dosage effects of X-linked genes as chicken does (Ayers et al. 2013).

Faster-Z in *Pelodiscus* Turtles Affects Genes Tied to Sexual Development

Z-linked genes in *Pelodiscus*, but not in *Apalone*, evolve faster than their autosomal orthologs in other amniotes (Table 1) revealing distinct evolutionary trajectories of these homologous sex chromosomes over ~95 million years of divergence (Badenhorst et al. 2013). This

Pelodiscus faster-Z pattern appears driven by positive selection which affected *Nf2* and *Sf3a1* (Supplementary Figure S1) at residues that change polarity and hydrophobicity relative to *Apalone*'s ortholog.

We investigated *Nf2* in more detail because 24 amino acids exhibited positive selection in *Pelodiscus*, many in functionally important sites (Pearson et al. 2000), and because *Nf2* participates in the hippo signaling pathway (Cockburn et al. 2013) which cross-talks with *Beta-catenin* (*Ctnnb1*) and members of the *Wnt* signaling pathway (Imajo et al. 2012) that help orchestrate vertebrate gonadogenesis (Kim et al. 2006; Liu et al. 2009). It is unknown whether positive selection acts directly on *Nf2* on PSI-Z, on neighboring genes that affect *Nf2* by linkage, or if *Nf2* accumulated mutations via relaxed purifying selection. We found significant sequence differences between *Pelodiscus* and *Apalone* in a ~212 kb gene block containing *Nf2*, *Sf3a1, Zmat5*, *Cabp7*, *Nipsnap1*, and *Ccdc157* (Kawagoshi et al. 2009), and not in its flanking regions (Table 2 and Supplementary Table S3). This gene block evolves 2.5× faster in *Pelodiscus* than in *Apalone*, suggestive of a selective sweep, and differences concentrate in an NF2 domain that can alter protein function (Echols et al. 2003). Interestingly, *Cabp7* and *Nipsnap1*, are linked to calcium ion binding and transient receptor potential (*Trp*) (Schoeber et al. 2008), two candidate regulators of gonadal development (Hanover et al. 2009) with sex specific roles (Wong et al. 2015) that are temperature-sensitive in eukaryotes (Shen et al. 2011), and are tied to TSD in *Alligator* (Yatsu et al. 2015) and painted turtles (Radhakrishnan et al. 2017). Positive selection in *Pelodiscus* cannot be attributed to GSD evolution in Trionychids because the same gene block in *Apalone* matches the rate of autosomal orthologs. Faster *Pelodiscus*-Z evolution ([Kawagoshi et al. 2009] and this study) may derive from reduced recombination (e.g. by deletions or inversions) (Montiel et al. 2017) which impairs mutation repair, also explaining the observed divergence between PSI-Z and ASP-Z. Successful hybridization of *Nf2* and *Sf3a1* probes onto PSI-Z but not PSI-W (Kawagoshi et al. 2009) support the deletion hypothesis.

Control Genes Evolve at Similar Rates Across Vertebrates, but Slower in Chicken

The control genes in our dataset were randomly chosen such that they are autosomal in all target species and have no housekeeping functions, and thus no difference in rate of evolution is expected between any given foreground and background species. This expectation held true in almost all species in our dataset, consistent with previous reports (Hughes and Mouchiroud 2001), except for chicken, whose control genes mimicked the slow evolution pattern seen in chicken sex-linked genes (Table 1). Agreeing with our findings, similar rates of molecular evolution among diverse vertebrates were reported (Hughes and Mouchiroud 2001), as well as overall lower rates in birds (Mindell et al. 1996). However, our results counter previous observations of similar rates in chicken and other vertebrates and faster molecular evolution in squamates (Hughes and Mouchiroud, 2001). Again, these discrepancies underscore that gene-specific effects exist and thus, generalizations based on a limited number of genes must be taken with caution.

Faster Molecular Evolution in TSD (Especially Turtles) than in GSD Vertebrates also Affects Genes Connected to the Sexual Development Network

Notably, orthologs of human sex-linked genes evolved faster in TSD species (*Chrysemys* turtle and *Alligator*) than in GSD species, and this results were robust to including or excluding the other TSD species in the background. Further, amniote sex-linked genes evolved faster in turtles relative to all other taxa, similar to immune function and musculoskeletal patterning genes previously reported despite the overall slower molecular evolution of turtle genomes (Shaffer et al. 2013). This observation underscores that genome-wide generalizations may obscure local gene phenomena, and highlight the importance of the chromosomal context driving these patterns. Further genome-wide studies are required to uncover patterns and exceptions among turtles.

One gene stood out in TSD turtles, *Tspan7*, a gene implicated in X-linked mental retardation in mammals, which exhibited extensive positive selection at ~20% of its residues in *Chrysemys*. We detected substantial 3D structure differences in TSPAN7 between *Chrysemys* and *Glyptemys*, the other Emydid turtle in our dataset (Figure 3B). Curiously, *Tspan7* is activated by the chimera of EWS-WT1, and *Wt1* regulates vertebrate gonadal formation (Pelletier et al. 1991; Spotila et al. 1998; Western et al. 2000; Wilhelm and Englert 2002) and is a candidate TSD master gene in *Chrysemys* (Valenzuela 2008).

Conclusion

Ours is the first examination of the molecular evolution of sex-linked sequences in turtles and reptiles relative to autosomes. Our approach using a small gene dataset across 11 amniotes, including 6 turtles, uncovered patterns not observed before. For instance, we detected faster-Z in *Pelodiscus* and slower-X in *Staurotypus* revealing significant effects of chromosomal context on molecular evolution. We also found that turtles vary in their molecular evolution even among closely related species that share a homologous sex chromosome system (*Pelodiscus* versus *Apalone*), revealing profound lineage-specific effects. The slower evolution of sex-linked sequences in the homologous *Staurotypus*-X and chicken-Z is intriguing and support the notion that certain ancestral chromosome possess properties that makes them good for sex (O'Meally et al. 2012; Montiel et al. 2016). Genome-level studies are needed before generalization can be made, but such efforts are increasingly facilitated by the availability of reptilian genomic resources in lizards (Alföldi et al. 2011), snakes (Castoe et al. 2013), alligators (St John et al. 2012) and turtles (Shaffer et al. 2013; Wang et al. 2013; Badenhorst et al. 2015; Tollis et al. 2017). This is critical to identify true patterns and exceptions, and to continue to illuminate the causes and consequences of sex chromosome evolution.

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Author Contributions

N.V. conceived of the project and wrote the manuscript. S.R. performed all bioinformatics analysis and wrote the manuscript.

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Data Availability

DNA sequences: Genbank accessions MF 686705-MF 686800 (Supplementary Table S3).

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