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Brief Communication

Population Genomic Analyses Confirm Male-Biased Mutation Rates in Snakes

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Abstract

Male-biased mutation rates occur in a diverse array of organisms. The ratio of male-to-female mutation rate may have major ramifications for evolution across the genome, and for sex-linked genes in particular. In ZW species, the Z chromosome is carried by males two-thirds of the time, leading to the prediction that male-biased mutation rates will have a disproportionate effect on the evolution of Z-linked genes relative to autosomes and the W chromosome. Colubroid snakes (including colubrids, elapids, and viperids) have ZW sex determination, yet male-biased mutation rates have not been well studied in this group. Here we analyze a population genomic dataset from rattlesnakes to quantify genetic variation within and genetic divergence between species. We use a new method for unbiased estimation of population genetic summary statistics to compare variation between the Z chromosome and autosomes and to calculate net nucleotide differentiation between species. We find evidence for a 2.03-fold greater mutation rate in male rattlesnakes relative to females, corresponding to an average μ_Z/μ_A ratio of 1.1. Our results from snakes are quantitatively similar to birds, suggesting that male-biased mutation rates may be a common feature across vertebrate lineages with ZW sex determination.

Subject Area: Molecular adaptation and selection
Key words: *Crotalus*, genomics, population genetics, sex chromosomes, speciation, ZW

Higher germline mutation rates in males than females are commonly observed across species (Li et al. 2002; Ellegren 2007). Most examples of male-biased mutation rates come from mammals (e.g., Chang et al. 1994; Lawson and Hewitt 2002; Li et al. 2002; Makova and Li 2002; Wilson et al. 2011) and birds (e.g., Ellegren and Fridolfsson 1997; Axelsson et al. 2004; Wang et al. 2014; Oyler-McCance et al. 2015), but they have also been observed in fish (Ellegren and Fridolfsson 2003), insects (Bachtrog 2008; Pinharanda et al. 2019), and plants (Whittle and Johnston 2002). While the causes of male mutation bias are debated and may vary among taxa (Ellegren 2007), a prevailing explanation is the greater number of germline cell divisions and replication errors in spermatogenesis compared to oogenesis, leading to the view that evolution is "male-driven" (Haldane 1935; Miyata et al. 1987).

Male-driven evolution is expected to have important consequences for substitution rates across the genome, especially when mutations are sex-linked and subject to purifying or positive selection (Kirkpatrick and Hall 2004). Indeed, the influence of male-biased mutation rates may be most pronounced on sex chromosomes depending on the pattern of inheritance and type of sex determination.

For example, the Y chromosome is carried only in males in XY species and is therefore expected to exhibit the fastest rates of evolution when mutation is male-biased. Similarly, with male-biased mutation the Z chromosome of ZW species may experience faster substitution rates than autosomes because the Z is in males 2/3 of the time. Hemizygosity of the Z in females also means that recessive or partially recessive mutations will be directly exposed to selection, potentially allowing for more efficient selection on the Z chromosome (Charlesworth et al. 1987; Irwin 2018). In this case, an excess of new beneficial mutations from males could result in higher rates of adaptive evolution on the Z chromosome. The lower effective population size (and therefore greater genetic drift) on the Z has also led to the conclusion that "faster-Z" evolution could occur primarily through an accumulation of slightly deleterious mutations (Vicoso et al. 2008; Mank et al. 2010). Male bias in the mutation rate, therefore, has the potential to drive "faster-Z" evolution under a variety of scenarios, including variation in the efficiency of selection on the Z chromosome.

While male-biased mutation is most extensively documented in the XY system of mammals, a large body of evidence has also accumulated for male-biased mutation rates in the ZW system of birds, confirming that male-specific mutational biases occur independently in male- and female-heterogametic systems (Wilson and Makova 2011). Previous estimates of the male-to-female mutation rate ratio vary widely across bird species (i.e., 1.8- to 6.5-fold more mutations in males; Ellegren and Fridolfsson 1997; Carmichael et al. 2000). Recent estimates based on genomic sequence data from a panel of 45 bird species suggest a more conservative range (i.e., 1.6–3.8; Wang et al. 2014), with an average ~2-fold male-to-female mutation rate ratio (Oyler-McCance et al. 2015; Irwin 2018). Data from birds highlight the considerable variation in species-specific mutation biases, similar to the range observed in mammals (Wilson and Makova 2011), suggesting that other vertebrate groups may also exhibit similar variation in male mutation bias.

Colubroid snakes represent a morphologically and taxonomically diverse radiation (3161 species; Uetz et al. 2020) that possess ZW sex chromosomes that evolved independently of bird ZW chromosomes (Matsubara et al. 2006). Compared to XY mammals and ZW birds, snakes are uniquely valuable for studying male-driven evolution because there is considerable variation in degeneration between Z and W chromosomes among colubroid species (Ohno 1967; Beçak and Beçak 1969; Matsubara et al. 2006). Variation in the degree of W degeneration is intriguing because it is predicted to result in variation in the impact of sex-biased mutational processes on genetic diversity across different regions of the sex chromosomes. The unique and variable colubroid sex chromosome system and potential for male-biased mutation therefore present a valuable opportunity for comparison to the ZW system of birds. Such comparisons may reveal important similarities and distinctions between 2 independently evolved systems with female heterogamety. Compared to birds, however, remarkably little is known about male-biased mutation in snakes. To our knowledge, only a single study has examined snakes for evidence for male-biased mutation rates (Vicoso et al. 2013). Based on analysis of synonymous substitution rates between 2 very distantly related snakes (~57.5 MYA; Kumar et al. 2017), a garter snake (*Thamnophis elegans*), and pygmy rattlesnake (*Sistrurus catenatus*), this study estimated a 1.8-fold male-to-female mutation rate ratio (Vicoso et al. 2013). While this estimate provides evidence for snake male mutation bias, its usefulness as a general estimate for snakes may be limited because the signal for biased mutation rates was averaged across tens of millions of years of divergence between 2 distantly related species that also have different degrees of degeneration between Z and W chromosomes (Matsubara et al. 2006). It is, therefore, unclear if this estimate accurately represents the male-biased mutation rate of each lineage and of colubroids more broadly. Further, the estimated mutation rate ratio was derived from synonymous sites within coding regions, which can be readily biased by saturation at such evolutionary divergences. This type of inference also assumes that all sequence differences between species are fixed, which may bias the resulting estimate.

We were motivated to confirm male-biased germline mutation rates in snakes using more robust population genomic data from several species, using the rattlesnake genus *Crotalus* as an example. *Crotalus* species occur in the Americas where they inhabit warm temperate, desert, and tropical regions (Campbell and Lamar 2004). Two clades native to North America comprise the "diamondback rattlesnakes" (the western diamondback (*Crotalus atrox*) and red diamondback (*C. ruber*)), and the Western rattlesnake species complex, including the Northern Pacific rattlesnake (*C. oreganus oreganus*) and the prairie rattlesnake (*C. viridis viridis*). These 2 clades diverged from a common ancestor nearly 6 million years ago (MYA), with pairs of species in each clade splitting roughly 3 MYA (Figure 1; Schield et al. 2020). This system thus provides a framework for multiple independent comparisons between species across similar timescales. In this study, we generate and analyze genome resequencing data for these species to estimate population genetic diversity and divergence statistics. We compare sequence divergence on the Z chromosome and autosomes using an evolutionary approach that has been applied across a variety of systems and degrees of divergence (i.e., tens to hundreds of millions of years; e.g., Axelsson et al. 2004; Goetting-Minesky and Makova 2006; Vicoso et al. 2008, 2013). An advantage of this method is that it is more feasible for studying natural populations of non-model organisms than estimating germline mutation rates across pedigrees, for example. Using this evolutionary approach, we estimate ratios of male-to-female germline mutation rate (α_m) and Z-to-autosomal mutation rate (μ_Z/μ_A) based on genome-wide estimates of population genetic variation and interpret our results in the context of other ZW species (e.g., birds).

Methods

We sequenced the genomes of males (ZZ) from the rattlesnake species described above, including *C. atrox* (n = 1), *C. ruber* (n = 1), *C. oreganus* (n = 15), and *C. viridis* (n = 17) (Supplementary Table S1). A total of 19 samples were generated and analyzed previously (Schield and Castoe 2020; Schield et al. 2020), and 15 were newly sequenced for this study (Supplementary Table S1). Individual *C. atrox* and *C. ruber* were sequenced as outgroup taxa to facilitate downstream analyses between "ingroup" population data from *C. viridis* and *C. oreganus*. All procedures using animals or animal tissue were performed according to the University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) protocols 0901C-SM-MLChick-12 and 1302D-SM-S-16. For samples generated in this study, DNA was extracted using standard phenol-chloroform-isoamyl extractions from blood samples preserved in DNA lysis buffer. Genomic libraries from purified DNA were prepared using Illumina Nextera Flex kits and sequenced on Illumina NovaSeq 6000 lanes using 150 bp paired-end reads at a mean coverage of 34× assuming a 1.5 Gbp genome size (Schield et al. 2020). We quality-filtered reads using Trimmomatic v0.39 (Bolger et al. 2014) with the settings LEADING:20 TRAILING:20

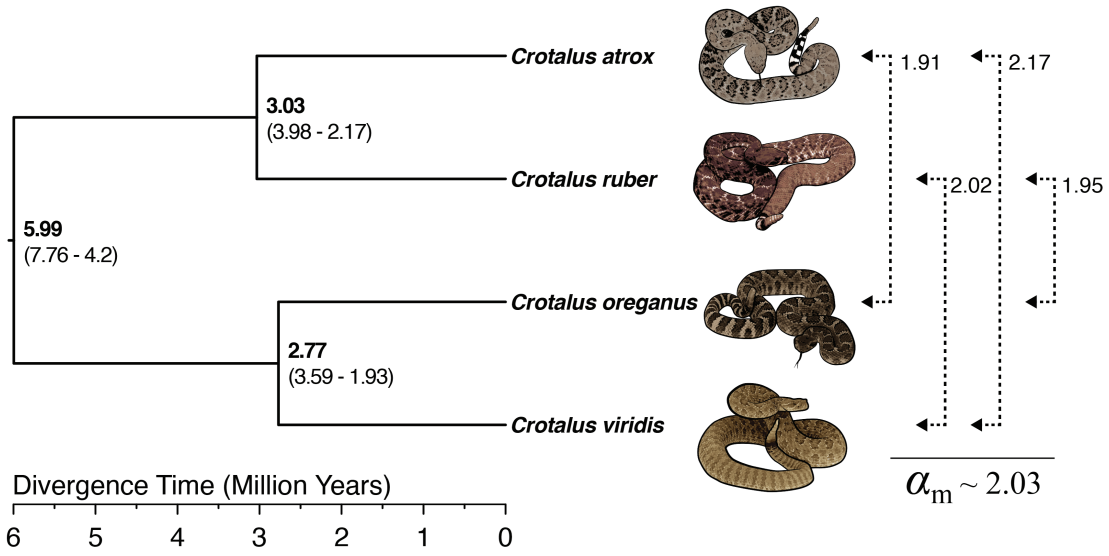

Figure 1. Phylogeny of rattlesnake species in the current study and inferred ratios of male-to-female mutation rate (αm). Values at node positions in the phylogenetic tree show divergence times and 95% posterior density ranges in millions of years redrawn from Schield et al. (2020). Dashed arrows and values to the right summarize pairwise divergence comparisons between species and associated αm estimates (the inferred average among species is ~2.03). The illustration for *C. ruber* was redrawn from an image courtesy of photographer Bryan Hughes.

MINLEN:32 AVGQUAL:30, then mapped filtered read data to the prairie rattlesnake reference genome (Schield et al. 2019) using bwa mem (Li and Durbin 2009) with default settings. An average of 97.1 ± 0.02% of reads mapped to the reference genome per sample.

Based on mapped read data, we generated an "all-sites" variant call file (VCF; Danecek et al. 2011), which encodes information for variant and invariant genotypes, using the GATK v4.0.8.1 best-practices workflow (McKenna et al. 2010; Van der Auwera et al. 2013). We first used GATK "HaplotypeCaller," specifying—ERC GVCF to generate a genomic VCF per individual, then called variant sites among the cohort of samples using GATK "GenotypeGVCFs" v3.8.1.0. We used GATK "VariantFiltration" to mask bases overlapping the prairie rattlesnake gene and repeat annotation, then used bcftools v1.20.2 (Li et al. 2009) to recode indels, masked genes, and repeats, and sites not meeting hard filters for depth and genotype quality (DP<5 and GQ<30) as missing genotypes. Filtering sites in genomic repeats and based on depth and quality filters was done to avoid spurious variant calls from influencing genetic diversity estimates. This filtering scheme resulted in variant sites sampled only from intergenic regions, similar to other studies that have used intergenic regions to estimate neutral mutation rates (Ellegren 2007). This procedure produced 53 124 088 intergenic variant sites from a total of 1 335 652 525 sites distributed across the genome for analysis. Notably, this sampling of sites represents several orders of magnitude greater variant information than previous estimates based on coding regions only.

We used pixy (Korunes and Samuk 2020; https://pixy.readthedocs.io) to calculate population genetic summary statistics across the genome using the filtered "all-sites" VCF generated above. Calculated statistics included average per-site heterozygosity (π; Nei and Li 1979), a measure of genetic diversity within lineages, and sequence divergence (d_{XY}) between lineages. Pixy uses full sequence information (i.e., variant and invariant sites) and explicitly accounts for missing data within and among sites when calculating summary statistics. π and d_{XY} are both most informative when full sequence data are analyzed (Cruickshank and Hahn 2014) and are sensitive to missing data (Nei and Roychoudhury 1974; Korunes and Samuk 2020); therefore, we expect the approach implemented in pixy to provide robust estimates of diversity and divergence. We ran pixy on each chromosome independently, estimating π and d_{XY} in 100 kb sliding windows. We filtered genotypes in the analysis using the options—variant_filter_expression "DP ≥ 5" and—invariant_filter_expression "DP ≥ 5."

We used the distributions of π and d_{XY} estimates to calculate net nucleotide differences (d_a) between species using the following equation adapted from Nei and Li (1979):

$$d_a = \quad d_{XY} - \frac{\pi_X + \pi_Y}{2} \quad \sim 2\mu T$$

We performed 4 independent comparisons, in each case calculating d_a between species from the diamondback and Western rattlesnake clades, respectively (e.g., *C. atrox* versus *C. viridis*, *C. ruber* versus *C. oreganus*, etc.). We calculated d_a to estimate the number of pairwise differences that have evolved since splitting from a common ancestor. Importantly, d_a is a relative measure of differences that have accumulated between species and approximates sequence divergence ($d = 2\ \mu T$; Nei 1972) conditional on the assumption that genetic diversity estimates for extant lineages (i.e., π) are representative of ancestral diversity (Cruickshank and Hahn 2014; Burri 2017). Therefore, to reduce the effects of lineage-specific diversity in any single rattlesnake species on d_a calculations, we also estimated a modified version of the d_a statistic, $d_{a\mathrm{ANC}}$, calculated as:

$$d_{a\mathrm{ANC}} = \quad d_{XY} - \frac{\pi_{C.atrox} + \pi_{C.ruber} + \pi_{C.viridis} + \pi_{C.oreganus}}{4}$$

Here, the approximation of ancestral diversity is based on mean current diversity in all species with the expectation that, if species-specific diversity levels are of large effect in d_a calculations (i.e., due to poor approximation of ancestral polymorphism), we will observe large differences in mutation rate ratio calculations based on d_a and $d_{a\mathrm{ANC}}$. We performed all d_a and $d_{a\mathrm{ANC}}$ calculations for autosomes and the Z chromosome separately, and for the Z chromosome omitted

the 7.2 Mb pseudoautosomal region (PAR) identified in Schield et al. (2019).

Comparing levels of sequence divergence between the Z chromosome and autosomes can reveal the relative contributions of new mutations from the sexes due to different inheritance patterns in males and females. We incorporated d_a values between species into the following equation from Miyata et al. (1987) to estimate the ratio of male-to-female mutation rate (α_m):

$$\alpha_m(Z/A) = (3Z/A - 2)/(4 - 3Z/A)$$

where d_a values for the Z and autosomes replace Z and A terms, respectively. We calculated α_m for each pairwise comparison of species and also an overall estimate for the group based on averaged d_a values for the Z and autosomes. We then used the averaged α_m ratio to calculate the ratio of Z-to-autosome mutation rate (μ_Z/μ_A) to evaluate how mutation bias and sex-linked inheritance influence the ratio of Z-linked-to-autosomal genetic diversity, following the equation used in Oyler-McCance et al. (2015) and based on Charlesworth et al. (1987) and Axelsson et al. (2004):

$$\mu_Z/\mu_A = (2/3\alpha_m + 1/3)/(1/2\alpha_m + 1/2).$$

Results and Discussion

Our estimates based on genome-wide population genetic statistics provide evidence of male-biased germline mutation in rattlesnakes, with an average α_m estimate of 2.08. When calculated after approximating ancestral polymorphism using current diversity estimates for all species (d_{aANC}), α_m was very similar (2.03). This indicates that d_a is robust to differences in species-specific diversity when averaged across multiple independent pairwise comparisons (also reviewed in Ellegren 2007). Further, this suggests that differential fixation of ancestral polymorphism contributes minimally to divergence between the diamondback and Western rattlesnake clades, and that divergence has instead accumulated through new mutations since splitting from their common ancestor. Although α_m inferred from specific comparisons between species varied (range = 1.91 – 2.17), ratios were consistently greater than 1 (Figure 1; Table 1). Inferred μ_Z/μ_A ratios based on d_a and d_{aANC} were 1.106 and 1.101, respectively, indicating that even α_m greater than 2 has a modest effect on relative Z chromosome and autosomal substitution rates. Nonetheless, the average ratio of Z-to-autosome π (π_Z/π_A) after correcting for male mutation bias was 0.69, versus 0.63 without correction. We note that, with the exception of *C. ruber* (which had very low genetic diversity overall; mean π = 0.00094), π_Z/π_A fell below the theoretically predicted 3/4 ratio after correcting for male-biased mutation on the Z (Table 2). This result is consistent with reduced Z-linked diversity due to excess variance in male reproductive success relative to females, a likely consequence of combined polygynandry, sperm storage, and sperm competition in rattlesnakes (Booth and Schuett 2011; Clark et al. 2014). In birds, variation in sex-linked diversity ratios has been attributed in part to different mating strategies (Corl and Ellegren 2012; Oyler-McCance et al. 2015; Irwin 2018). Snakes also have a wide variety of mating strategies (Shine 2003), which could contribute to similar interspecific variation in sex-linked versus autosomal genetic diversity. A faster accumulation of mutations on the Z chromosome (i.e., ~10% faster than autosomes) due to male mutation bias suggests that the Z may be a hotspot for genes and regulatory sequences underlying sexual dimorphism, under sexual selection, or with sexually antagonistic effects (e.g., Charlesworth et al. 1987; Albert and Otto 2005; Vicoso et al. 2008). Broadly, this suggests that the elevated mutation frequency on the Z may play a prominent role in colubroid speciation.

Our estimates of α_m confirm the presence of male mutation bias in rattlesnakes and, together with the previous point estimate from Vicoso et al. (2013), suggest that male-biased mutation rates are a general feature of ZW snake evolution. As noted above, the previous estimate is based on divergence at synonymous sites (d_S) with the assumption that any differences are fixed between species, and is averaged between 2 species with deep divergence (~57.5 MYA; Kumar et al. 2017). We suggest that the population genomic approach applied here is more appropriate for estimating mutation bias because it incorporates variation across individuals when evaluating divergence between lineages and does not assume that differences are fixed. Our approach also has the advantage of sampling millions of sites in intergenic regions of the genome and should therefore provide more robust estimates of male mutation bias. The resultant estimates for α_m from our *Crotalus* data are higher than the previous estimate based on d_S between distantly related species (i.e., α_m = 2.03 versus 1.8), suggesting that the previous ratio underestimates male-biased mutation in *Crotalus*, and potentially other colubroids with highly differentiated sex chromosomes. It remains an open question if rates are indeed lower in other snake lineages, such as *Thamnophis* and *Sistrurus* (as suggested by Vicoso et al. 2013). Similar analyses of population genomic data for *Sistrurus* and *Thamnophis* species using the approach applied here would be useful in testing if male mutation bias is genuinely lower in these lineages than in *Crotalus*, or if other aspects of previous estimates resulted in systematic underestimation of these rates. Our findings further suggest that male-to-female mutation rate ratios in ZW snakes are remarkably similar to the average estimate for birds (i.e., ~2; Irwin 2018) despite having non-homologous sex chromosomes. However, the considerable

Table 1. Population genetic summary statistics, male-to-female mutation rate ratios (αm), and Z-to-autosomal mutation rate ratios (μ_Z/μ_A) estimated for the rattlesnake species in the current study

Species comparison	d_{XY} Z / d_{XY} Auto	d_a Z / d_a Auto	d_{aANC} Z / d_{aANC} Auto	α_m	α_m*	μ_Z/μ_A	μ_Z/μ_A*
C. atrox vs. *C. oreganus*	0.00644 / 0.00670	0.00494 / 0.00427	0.00506 / 0.00459	2.797494	1.914121	1.106	1.101
C. ruber vs. *C. oreganus*	0.00606 / 0.00635	0.00482 / 0.00456	0.00469 / 0.00424	1.416888	1.952346		
C. atrox vs. *C. viridis*	0.00825 / 0.00823	0.00664 / 0.00563	0.00687 / 0.00612	3.355008	2.174196		
C. ruber vs. *C. viridis*	0.00779 / 0.00788	0.00654 / 0.00609	0.00642 / 0.00577	1.581807	2.02376		
Mean	0.00713 / 0.00729	0.00574 / 0.00513	0.00576 / 0.00518	2.080	2.03		

d_{aANC} = net nucleotide differentiation when ancestral polymorphism is approximated using estimates from all species; α_m* = male-to-female mutation rate ratio calculated using d_{aANC}; μ_Z/μ_A* = Z-to-autosome mutation rate ratio calculated using α_m*.

Table 2. Within-population genetic diversity, π, and ratios of Z chromosome and autosomal diversity with and without correction for male-biased mutation rate, αm

Species	π_Z	π_{Auto}	π_Z/π_A	$\pi_Z/\pi_A{}^*$
C. atrox	0.00164	0.00258	0.63512	0.57738
C. ruber	0.00092	0.00096	0.96316	0.87560
C. oreganus	0.00136	0.00229	0.59293	0.53903
C. viridis	0.00157	0.00263	0.59636	0.54215
Mean	0.00137	0.00211	0.69689	0.63354

$\pi_Z/\pi_A{}^*$ = Z-to-autosomal genetic diversity ratio after correction for male-biased mutation rate.

range of α_m values inferred across bird species cautions that a broader survey of snake species may reveal greater variation in male mutation bias among lineages.

There are several potential links between rattlesnake life history and male mutation bias that warrant further exploration. For example, long-lived species that reproduce at later ages and have several bouts of reproduction will experience higher numbers of cell divisions in spermatogenesis, contributing to a higher α_m than short-lived species that reproduce only once (Ellegren 2007). Sperm competition can also drive higher rates of sperm production and therefore cell divisions in spermatogenesis. Rattlesnakes, and colubroid snakes in general, vary considerably in reproductive and life-history traits (e.g., age at first reproduction, annual versus biennial reproduction in females; Campbell and Lamar 2004), and sperm competition is documented in multiple species (Tourmente et al. 2009; Clark et al. 2014). While direct estimates of differences in rates of spermatogenesis and oogenesis in rattlesnakes are lacking, the existence of variation in reproductive life history traits argues that such variation may indeed contribute to variation in α_m among species. The intensity of sexual selection on males could also impact relative rates of male versus female germline mutations and the relationship between sex-linked and autosomal genetic diversity. Rattlesnakes often exhibit sexual size dimorphism, with larger male body size presumably under sexual selection for access to females through male-male combat (Shine 1994; Hendry et al. 2014). Operational sex ratios for rattlesnake species are also typically skewed toward males (Duvall et al. 1992), imposing stronger sexual selection on males and greater variance in male reproductive success. Future studies evaluating how variation in life-history traits across species corresponds with α_m would be valuable for identifying factors that play a prominent role in the evolution of male mutational bias. In the case of sperm competition, for example, it would be interesting to examine whether higher mutation rates in males are a byproduct of selection for increased spermatogenesis.

As W chromosome reference sequences and associated population genomic resources for snakes become available, an intriguing future avenue of research related to male mutation bias would be to perform comparisons that involve the W chromosome, specifically (i.e., Z-to-W and W-to-autosome ratios). Miyata et al. (1987) devised 3 formulas for calculating α_m that are expected to be exactly equivalent if male-biased mutation rates are the sole cause of substitution rate variation between chromosomes:

$$\alpha_m(Z/A) = (3Z/A - 2)/(4 - 3Z/A)$$

$$\alpha_m(Z/W) = (3Z/W - 1)/2$$

$$\alpha_m(A/W) = (2A/W - 1)$$

Differences in α_m estimates from these formulas would indicate that variables independent of replication errors contribute to substitution rate variation between chromosomes, such as recombination (Wilson and Makova 2011). Thus, the availability of additional W chromosome sequence resources could be valuable for testing the relative roles of replication versus other factors in shaping male-biased mutation variation. Rattlesnakes in particular have considerable recombination rate variation within and among chromosomes (Schield et al. 2020), thus substitution rate variation across the genome as a consequence of male-biased mutation rates and recombination is reasonable. Indeed, considering the substantial variation in heteromorphism between Z and W chromosomes and associated variation in the size of recombining pseudoautosomal regions among colubroid snake species (Matsubara et al. 2006; Vicoso et al. 2013; Gamble et al. 2017), colubroids would provide a valuable model for studying how recombination rate variation, male mutation bias, and sex linkage influence rates of evolution.

As a final consideration, it is possible that mechanisms have evolved to balance the high relative frequency of male germline mutations on the Z chromosome in snakes. Although our mutation rate estimates are from intergenic regions, we expect that higher background mutation rates apply to all non-PAR regions of the Z chromosome, including coding regions. Considering this, more efficient selection on Z-linked genes (e.g., Vicoso et al. 2008) predicts a higher likelihood that new beneficial mutations will reach fixation. Conversely, higher efficiency of selection on the Z against deleterious mutations could have a compensatory effect against mutational input (Charlesworth et al. 1987), resulting in an adaptive reduction of the Z chromosome mutation rate. In this case, we would expect to observe a lower Z-linked mutation rate than under null expectations (Axelsson et al. 2004). While our data do not allow us to make comparisons with the W chromosome necessary to evaluate if there is a Z-specific reduction in substitution rate, our results are generally consistent with the male-biased mutation predictions of Miyata et al. (1987). Accordingly, if there is a compensatory effect of selection on the Z chromosome, its effects may be quite weak, similar to the weak effect inferred in birds (Axelsson et al. 2004). Once W-linked sequences for rattlesnakes and other ZW snake species become available, direct comparisons of sequence divergence between both sex chromosomes and the autosomes could provide valuable information on the relative role of male mutation bias in genome-wide variation. As greater genomic resources for diverse organisms accumulate, the approach for inferring male mutation rates developed here can be applied to evaluate evidence of sex-biased mutation rates in closely related groups of species based on population genetic summary statistics that have existed for decades (e.g., Nei 1972; Nei and Li 1979; Miyata et al. 1987).

Supplementary Material

Supplementary material can be found at *Journal of Heredity* online.

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Conflict of Interest

The authors declare no conflicts of interest related to this work.

Data Availability

We have deposited materials supporting the conclusions of this work as follows:

- Whole-genome resequencing data: NCBI SRA accession PRJNA593834
- Analysis scripts: Github https://github.com/drewschield/male-biased_mutation_crotalus

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