RESEARCH ARTICLE SUMMARY

EVOLUTION

Genomic predisposition is associated with the direction of sex chromosome evolution

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Full article and list of author affiliations: https://doi.org/10.1126/science.aec3449

INTRODUCTION: Sex-determination systems are highly diverse across vertebrates, ranging from environmental sex determination (ESD) to genetic sex determination (GSD), with GSD typically taking the form of male heterogamety (XY) or female heterogamety (ZW). Sex chromosomes evolve rapidly within and between lineages, raising the question of why particular ancestral chromosomes are recurrently recruited as sex chromosomes.

RATIONALE: Although the processes underlying sex chromosome formation and differentiation have been studied extensively, factors associated with transitions between different sex-determination systems remain elusive. Theoretical models suggest that environmental instability may favor transitions from ESD to GSD, but empirical support is limited. It is also unclear why some lineages evolve XY systems whereas others evolve ZW systems. Geckos provide an unusually informative model for addressing these questions: The clade exhibits exceptional diversity in sex determination, including temperature-dependent sex determination (TSD) as well as multiple independently evolved XY and ZW systems at various stages of differentiation. We combined chromosome-level genome assemblies, phylogenetic reconstruction, and comparative transcriptomics to explore the interplay between environmental pressures, genomic architecture, and evolutionary constraints shaping sex chromosome evolution.

RESULTS: We produced chromosome-level genome assemblies for 19 gecko species, spanning all seven extant families and capturing TSD, XY, and ZW systems. Together with three published gecko genomes, ancestral karyotype reconstruction revealed that the 22 sex chromosome systems across 20 GSD species arose independently from 17 ancestral chromosomes. Despite this independence, their origins are nonrandom in time: Five of the 11 datable lineages began differentiating their sex chromosomes within a 7 million to 12 million year window, coinciding with the Middle Miocene Climatic Transition (MMCT). The direction of evolution is also nonrandom. Comparative transcriptomic analysis of the oldest sex-differentiated regions (SDRs) showed that ancestral gene content is associated with the subsequent sex chromosome trajectory: Regions containing an excess of testis preferentially expressed genes (TPEGs) over the genomic background tend to evolve into ZW systems, whereas regions with reduced TPEG proportions tend to evolve into XY—a pattern observed across multiple independent amniote sex chromosome origins. Across gecko lineages, Y and W chromosomes exhibited age-dependent patterns of gene retention, and most geckos evolved partial dosage balance between sexes through up-regulation of sex-linked genes in the heterogametic sex.

CONCLUSION: Our results suggest that the repeated independent origins of sex chromosomes in geckos reflect the combined influence of external environmental context and intrinsic genomic content. Major climatic change may have created conditions favoring transitions from ESD to GSD, whereas preexisting differences in ancestral gene expression appear to be associated with whether male or female heterogamety subsequently evolves. Together, these findings indicate that sex chromosome evolution follows constrained pathways shaped by ancestral genomic predisposition and broader environmental context, rather than purely stochastic processes. □

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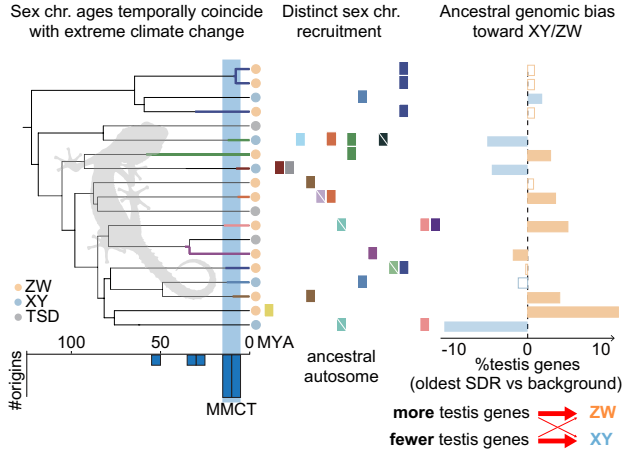

Constrained pathways of sex chromosome evolution. Most gecko sex chromosomes originated independently from different ancestral chromosomes. Five of 11 datable origins coincide near the MMCT [~10 million years ago (Mya)]. Regions ancestrally enriched in TPEGs tend to evolve into ZW systems, whereas regions depleted of such genes tend to evolve into XY—a pattern observed across multiple independent amniote sex chromosome origins. [Gecko silhouette is copyrighted by Stuart V. Nielsen]

EVOLUTION

Genomic predisposition is associated with the direction of sex chromosome evolution

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Sex chromosome evolution is among the most dynamic genomic innovations in vertebrates, yet why some lineages evolve XY while others evolve ZW remains unclear. Using chromosome-level genomes from 19 geckos, we found that gecko sex chromosomes originated independently from 16 ancestral chromosomes. Their origins are nonrandom in both time and direction. Five of 11 datable origins coincide near the Middle Miocene Climatic Transition (~10 million years ago). Ancestral gene content is associated with the direction of evolution, with testis-enriched regions tending to evolve into ZW and testis-depleted regions into XY, a pattern observed across multiple amniote sex chromosome origins. Geckos with genetic sex determination repeatedly evolve partial dosage balance through up-regulation in the heterogametic sex. Sex chromosome evolution thus follows constraints from ancestral genomic predisposition and environmental context.

Sex-determination systems are exceptionally diverse across vertebrates, ranging from environmental sex determination (ESD) to genetic sex determination (GSD). Transitions between these systems have occurred repeatedly, yet factors underlying these transitions remain incompletely understood (*1*–*3*). Although theoretical models predict that ESD should evolve under stable environmental conditions and GSD under fluctuating conditions (*1*), empirical support is limited. Furthermore, when GSD does evolve, the factors determining whether male heterogamety (XY) or female heterogamety (ZW) systems emerge are largely unknown (*4*). Key unresolved issues include what triggers GSD evolution (*1*, *5*), why particular genomic regions are repeatedly recruited as sex chromosomes (*6*–*8*), and what determines the evolutionary direction toward XY or ZW (*9*, *10*).

The genomic consequences of sex chromosome evolution extend far beyond sex determination itself, influencing fundamental aspects of gene regulation, dosage compensation, and genome organization (*11*–*14*). An unresolved question is whether the genomic content of ancestral regions influences their evolutionary fate as sex chromosomes, or whether sex-biased gene expression patterns emerge only after sex chromosome establishment (*2*, *4*, *12*). Moreover, the mechanisms governing gene survival during the progressive degeneration of Y and W chromosomes vary drastically across species, with some retaining most ancestral genes while others undergo extensive gene loss (*2*, *15*, *16*).

Geckos exhibit pronounced variation in sex-determination systems, including temperature-dependent sex determination and both XY and ZW genetic systems at various stages of differentiation (*17*–*19*). This diversity spans from recently evolved, homomorphic sex chromosomes to ancient, highly differentiated systems (*20*). The phylogenetic distribution of different sex-determination systems in geckos suggests multiple independent transitions between ESD and GSD, providing opportunities to test hypotheses about the ecological and genomic factors driving sex chromosome evolution (*18*, *19*). In this study, we analyzed 19 gecko species, representing ~70% of currently known independent sex chromosome origins across all gecko families, capturing the documented diversity of sex-determination mechanisms (*18*, *19*). By combining chromosome-level genome assemblies with comparative transcriptomic analyses across this natural diversity, we explored the environmental pressures, genomic architecture, and evolutionary constraints that shape sex chromosome evolution.

Chromosome-level genome assembly and sex chromosome identification across gecko lineages

We generated high-quality chromosome-level assemblies for 19 gecko species representing all seven families of geckos (infraorder Gekkota) using an integrated approach combining PacBio long reads (continuous long reads or circular consensus sequencing), 10X Genomics link reads [or single-tube long fragment reads (*21*)], and Hi-C sequencing technologies (Fig. 1 and tables S1 to S3). This dataset provides high-quality genomic coverage across the three major vertebrate sex-determination systems: temperature-dependent sex determination (TSD; 3 species), XY (5 species), and ZW (11 species), encompassing the full spectrum of sex chromosome differentiation stages from homomorphic to highly heteromorphic states (*17*–*19*). Compared with the published gecko assemblies, our newly generated dataset exhibits marked improvement in both sequence continuity and completeness (fig. S1 and table S3), with an average scaffold N50 of 132.83 Mb, BUSCO completeness of 93.74%, and a gap ratio of 0.08% (table S3). Approximately 96% of each assembly was assigned to chromosomes (table S3). Genome size ranges from 1.6 to 2.5 Gb across most species except for *Nephrurus levis* (~3 Gb), whose expanded size primarily results from extensive proliferation of long terminal repeats and long interspersed nuclear elements (fig. S2 and tables S4 and S5).

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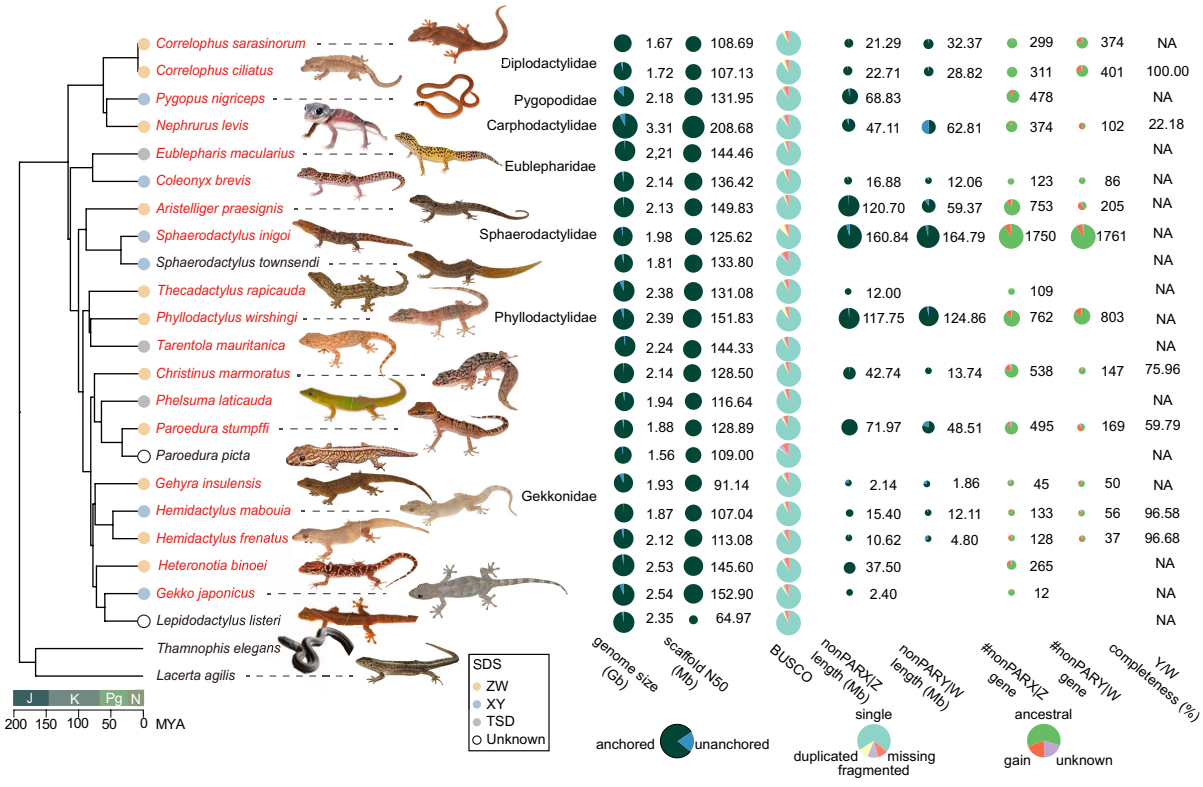

Fig. 1. Gecko genome assemblies, phylogeny, and sex-linked sequence dataset. Genomic phylogeny of gecko with divergence times as branch lengths. J, Jurassic; K, Cretaceous; Pg, Paleogene; N, Neogene. Species name color: red, generated in this study; black, published. The sex-determination systems (SDS) are also shown at the leaf node: blue, XY; orange, ZW; gray; TSD. Bubble plots illustrate key assembly and sex chromosome parameters. The bubble sizes have been scaled to each panel and are not comparable across panels. [All gecko photos are copyrighted by Stuart V. Nielsen, Aaron H. Griffing, or Tony Gamble, except *Lepidodactylus listeri* (copyrighted by Luis Mata under CC BY-NC 4.0) and *Thamnophis elegans* (copyrighted by Inklein under CC BY-SA 3.0)]

We identified putative X and Y (or Z and W) chromosomes and their sex-differentiated regions (SDRs) in 12 out of 16 GSD species through integrated analysis of sex-biased sequencing depth patterns and Hi-C interaction signals (table S6 and fig. S3). For three species with homomorphic sex chromosomes (*22*–*24*), *Thecadactylus rapicauda*, *Heteronotia binoei*, and *Gekko japonicus*, we failed to resolve both sex chromosomes (figs. S4 to S6), probably because of extremely low sequence divergence between the sex chromosome pair. Nevertheless, we identified their SDR with resequencing or restriction site–associated DNA sequencing data of multiple male and female individuals (methods and figs. S4 to S6). In *Pygopus nigriceps*, which has an XX/XY system (*25*), we assembled the complete X chromosome and identified its SDR and pseudoautosomal region (PAR) (fig. S7) but discovered no detectable male-specific sequence in either genomic or transcriptomic data (supplementary text), suggesting that the Y-SDR has undergone extreme degeneration. Hi-C contact maps revealed evidence of multiple sex chromosome systems in two species, *Sphaerodactylus inigoi* (XX/XY1Y2) and *Coleonyx brevis* (X1X1X2X2/X1X2Y) (fig. S8), likely derived from sex chromosome–autosome fusions (*26*–*28*).

The quality and biological relevance of our sex chromosome assemblies were validated by multiple lines of evidence. Our assembled sex chromosomes have recovered known sex-linked markers from previous cytogenetic and molecular studies in 10 species (*18*, *28*–*30*) (table S6). The sex-linked sequence assemblies are highly continuous, with >95% of X- or Z-linked sequences assembled into single scaffolds for 15 GSD species. Although Y- or W-linked sequences appeared relatively fragmented owing to repetitive element complexity, >90% of sequences were still assembled into a single scaffold in 8 out of the 16 GSD species (Fig. 1 and table S6). Comparison with published karyotypes (*22*–*24*, *31*–*36*) further confirmed the high completeness of our Y/W dataset (Fig. 1 and table S7).

Annotation of these high-quality assemblies, based on homologous protein evidence and RNA sequencing data from brain, eye, tail, and gonadal tissues of both sexes, yielded an average of 20,319 protein-coding genes per species (table S8). The number of identified gametolog pairs varied drastically across species, ranging from only 14 in *Hemidactylus frenatus* to 1506 in *Sphaerodactylus inigoi*, with the ratio of gametolog pairs to total X/Z-SDR genes spanning from 11.23% (*Nephrurus levis*) to >90% (genus *Correlophus*) (tables S9 and S10).

To establish the evolutionary framework for comparative analysis, we constructed a phylogenetic tree using whole-genome alignments from our 19 chromosome-level assemblies along with three published gecko genomes (*Sphaerodactylus townsendi*, *Paroedura picta*, and *Lepidodactylus listeri*) and two outgroups (*Lacerta agilis* and *Thamnophis elegans*). Our phylogeny based on 89.84 Mb whole-genome alignment (table S11) strongly supports (local posterior probability = 1.00) established gecko relationships while providing precise divergence time estimates through fossil calibration (*37*, *38*) (Fig. 1 and fig. S9). This analysis indicates that geckos diverged from all other snakes and lizards about 191 million years ago (Mya), followed by rapid radiation during the late Cretaceous to early Paleogene (Fig. 1).

Independent origins but limited homology of sex chromosomes in geckos

The diversity of sex-determination systems and sex chromosomes across geckos has long suggested extensive evolutionary lability (*18*, *19*, *39*).

Previous studies have indicated that sex chromosomes originated independently multiple times from TSD ancestors within this clade rather than resulting from frequent sex chromosome turnover events (*3*). However, the extent to which these independent origins are truly random, whether particular chromosomes are preferentially reused, and how much homology is retained at the level of SDRs have remained unresolved.

We inferred the ancestral karyotype evolution of geckos using the 22 chromosome-level gecko assemblies with two outgroup species, with *Correlophus sarasinorum* as the reference. Our reconstruction revealed that the gecko most recent common ancestor had $2n = 38$ chromosomes (Fig. 2A and tables S11 to S13), confirming the ancestral karyotype inferred from cytogenetic data (*40*). This karyotype configuration remains largely intact in extant species such as the genus *Correlophus* and *Nephrurus levis*, which show no detectable interchromosomal rearrangements relative to the ancestral state (figs. S10 and S11). Consistent with earlier work, our comparative genomic analyses provide strong evidence that gecko sex chromosomes originated independently across lineages. Incorporating three additional published GSD geckos with available SDR data (*Saltuarius cornutus*, *Coleonyx elegans*, and *Cyrtodactylus pharbaungensis*) (*19*, *41*, *42*), we found that the 22 sex chromosome systems across 20 GSD species

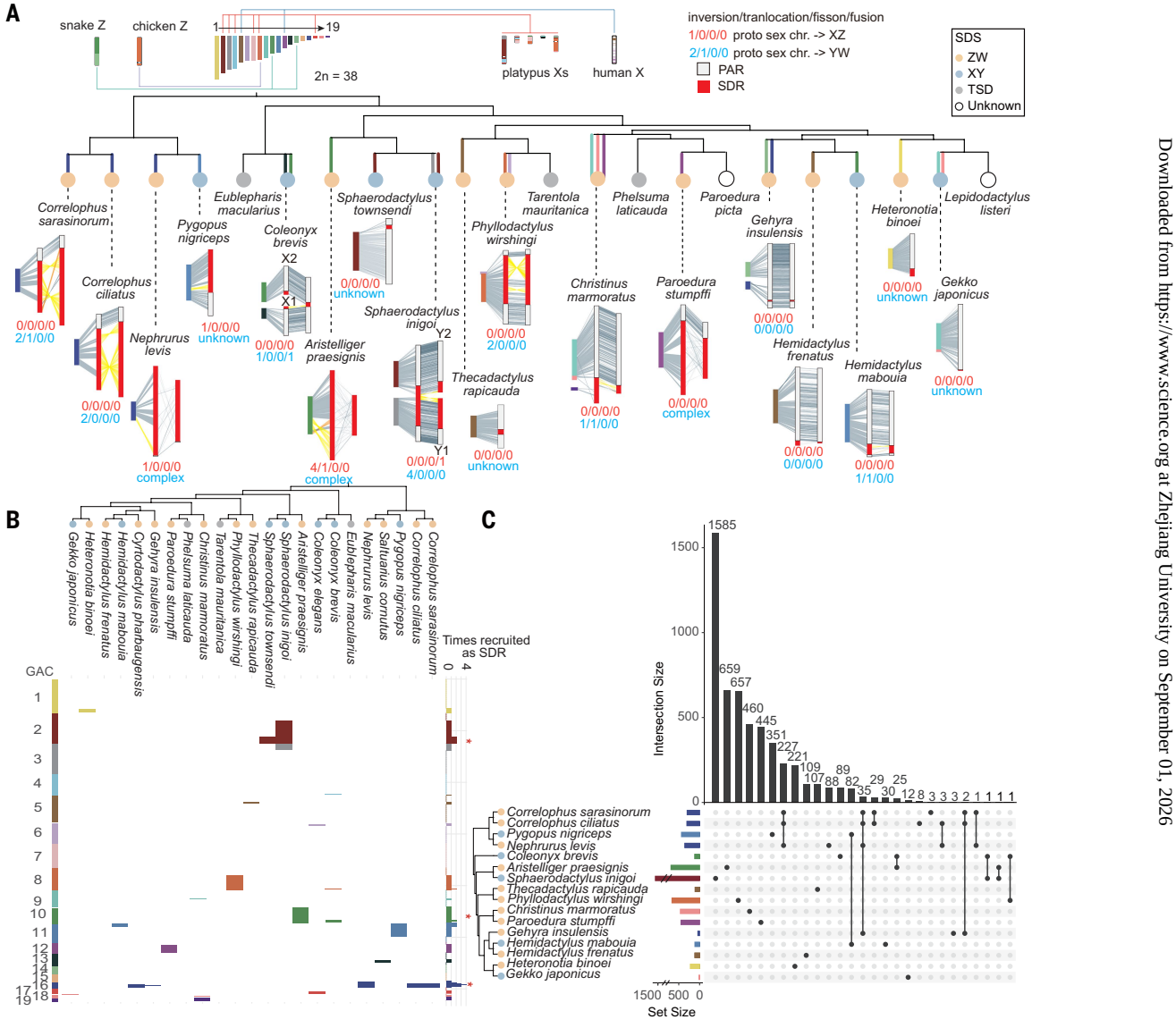

Fig. 2. Repeated independent origins of gecko sex chromosomes. (**A**) Reconstructed ancestral karyotype of Gekkota and the evolution of sex chromosomes in each GSD species. Branch to tip is color coded for each GSD species according to the GACs that evolved into sex chromosomes. For most GSD species, three chromosomes are shown (from left to right): proto sex chromosome, X or Z, and Y or W. In species with lowly differentiated sex chromosomes, proto sex chromosome (left) and a merged representation of the sex chromosomes (XY or ZW) (right) is shown. Chromosomal rearrangements occurred during sex chromosome evolution are highlighted in yellow (inversion) and orange (translocation). The number of inferred rearrangements from proto sex chromosome to current sex chromosome(s) are shown in the order of inversion, translocation, fission, and fusion. Homology between sex chromosomes in human, platypus, chicken, and snake and GACs are shown at the top. (**B**) Distribution of conserved blocks detected as SDR in all studied geckos. Boxes are color coded by GAC as in (A). The number of times a conserved block is used as SDR is indicated on the right. Conserved blocks with significant nonrandomness (before FDR correction) are highlighted by red asterisks. When blocks are small and in close proximity to each other, we use only one asterisk to mark a region in the genome that is constituted by multiple continuous significant blocks. (**C**) Upset plot for one-to-one ortholog gene on X- or Z-linked SDR. The color of the bar plot on the left corresponds to the GACs.

are derived from 17 different gecko ancestral chromosomes (GACs) (Fig. 2B and fig. S12). This pattern of repeated sex chromosome recruitment across gecko lineages confirms the widespread independence of sex chromosome origins established by previous studies while substantially extending our understanding through chromosome-scale assemblies and explicit SDR identification across a broader phylogenetic range.

Within this overall pattern of independent origins, sex chromosome recruitment exhibits some genomic predisposition effects (Fig. 2B). For example, the GAC16 independently evolved into a ZZ/ZW system in four separate lineages: *Correlophus* spp., *Nephrurus levis*, *Cyrtodactylus pharbaungensis*, and *Gehyra insulensis*. Whereas *Nephrurus levis* adopted a ZZ/ZW system from GAC16, its sister lineage *Pygopus nigriceps* independently established an XX/XY system from GAC11. Similarly, GAC11 was also co-opted as an XX/XY system in the distantly related *Hemidactylus mabouia*, GAC2 independently evolved into a XX/XY system in both *Sphaerodactylus* species, and GAC5 convergently evolved into a ZZ/ZW system in both *Hemidactylus frenatus* and *Thecadactylus rapicauda*, although the latter underwent a fission event that retained only part of GAC5 as its sex chromosome (Fig. 2A). These findings echo the parallelism observed between *Gekko hokouensis* and birds, which use homologous ancestral chromosomes for sex determination (*43*). Moreover, we observed that the same GAC could give rise to sex chromosomes with opposite heterogamety patterns. For example, GAC10 evolved into ZZ/ZW in *Aristelliger praesignis* but XX/XY in *Coleonyx brevis* (fig. S12). Similar cases can be found between *Gekko japonicus* X and a part of *Christinus marmoratus* Z that originated from fusion of GAC9 and GAC18, and between *Coleonyx brevis* X2 and *Saltuarius cornutus* Z from GAC13.

Despite the broad independent sex chromosome recruitment with some predisposition effect at the chromosome level, the underlying SDRs remain largely nonhomologous (fig. S13). For instance, although the sex chromosome of both *Nephrurus levis* and *Correlophus* spp. evolved from GAC16, their PARs are located at opposite ends of the chromosome. In *Aristelliger praesignis*, the SDR spans nearly the entire GAC10, whereas in *Coleonyx brevis*, the SDR covers only ~10% of the same ancestral chromosome, with the remainder functioning as PAR. The SDRs of *Hemidactylus mabouia* and *Pygopus nigriceps* overlap by only ~23% despite both originating from GAC11. In extreme cases, such as *Hemidactylus frenatus* versus *Thecadactylus rapicauda* and *Christinus marmoratus* versus *Gekko japonicus*, SDRs show no overlap despite sharing the same ancestral chromosomal origin.

To rule out the possibility of sex chromosome turnovers involving different chromosomes, potentially caused by a new sex-determining mutation in a previously autosomal locus or the translocation of the ancestral sex-determining gene to another chromosome (*3*), we compared the sequence divergence and gene content between sex chromosomes across different species. Sex chromosome identity between X/Y (or Z/W) pairs varies considerably across species, ranging from 88.65 to 99.97% (Fig. 3A), with the most diverged sex chromosomes found in Z/W of *Paroedura stumpffi*, and the most similar in *Correlophus* spp. and *Heteronotia binoei* (>95% identity; Fig. 3A). All observed X/Y (or Z/W) sequence divergences fall well below the divergence level observed between each other's closest relatives in our sampling (fig. S14), excluding scenarios of the involvement of an ancestral sex chromosome followed by turnover to different chromosomes. Gene-level analysis provides additional support for independent origins. Most SDR genes in any given species correspond to autosomal genes in other gecko species, and when shared SDR genes do exist, they cluster by species rather than by sex chromosome identity (Fig. 2C, figs. S15 to S18, and tables S9 and S14 to S16). These phylogenetic patterns provide evidence against sex chromosome turnover scenarios and support the repeated independent recruitment model.

Our findings also address a critical methodological concern in testing theories of nonrandom sex chromosome evolution. Previous studies often treated entire chromosomes as analytical units without distinguishing SDRs from PARs (*8*, *44*–*46*), potentially obscuring evolutionary complexity as PARs retain autosomal-like characteristics including recombination and dosage effects (*47*). To overcome this limitation, we used evolutionarily conserved syntenic blocks as units, providing unprecedented resolution for testing nonrandom recruitment patterns. Across the 23 species with reliably identified SDRs, we observed 98 conserved syntenic blocks that independently and repeatedly (twice or more) evolved into SDRs. When each block was evaluated against its own length-corrected null distribution, only 12 of 98 showed excess recruitment before correction for multiple testing, and none remained significant after false discovery rate (FDR) correction (table S13). This indicates that recruitment of specific genomic regions is generally weak or absent at the syntenic block level. When we repeated this analysis using whole chromosomes as units, we observed significant enrichment of GAC16 but also detected false positives on GAC18, where SDRs between *Christinus marmoratus* and *Gekko japonicus* show no actual homology (fig. S13).

Temporal distribution of sex chromosome divergence in geckos

The numerous independent origins of sex chromosomes across gecko lineages present a natural experiment for investigating the evolutionary forces that drive transitions from ancestral ESD to GSD (*11*, *15*). To explore whether such innovations are stochastic or driven by shared environmental pressures, we estimated the timing of sex chromosome divergence across all GSD gecko species using the substitution rate of the noncoding region before and after sex chromosome differentiation, after correcting for male mutation bias in each lineage (methods). We found that the sex chromosome ages vary widely, ranging from 5.90 million years (Myr) in *Phyllodactylus wirshingi* to 54.45 Myr in *Aristelliger praesignis* (Fig. 3A, figs. S19 to S22, and tables S17 and S18). To validate our divergence time estimates, we independently estimated stratum ages using fourfold degenerate sites, obtaining highly consistent results [Pearson's correlation coefficient (r) = 0.97, $P = 6.99 \times 10^{-13}$; table S18]. We also inferred de novo mutation rate from the whole-genome alignment using RAxML (Randomized Axelerated Maximum Likelihood), and calculated age by dividing the X/Y (or Z/W) divergence with the inferred de novo mutation rate after correcting for male mutation bias (methods), obtaining highly consistent results (Pearson's $r = 0.99$, $P = 3.98\times 10^{-16}$; table S18).

Despite originating from different ancestral chromosomes and evolving independently across distantly related gecko lineages that diverged 7.33 million to 87.62 million years ago (Fig. 1), sex chromosomes in many gecko species began diverging within a relatively narrow temporal window (Fig. 3A). Specifically, 5 out of 11 species with GSD showed sex chromosome divergence times clustering between 7 and 12 Mya, with an average of 9.55 Mya (Fig. 3A and table S18). To test whether this temporal concentration deviates from random expectation, we performed a phylogenetically constrained Monte Carlo simulation (methods). Null replicates rarely produced as many origins in the 7- to 12-Mya window as observed ($P < 0.01$; fig. S23), supporting a nonrandom temporal distribution. Although this clustering does not represent a universal pattern across geckos, it does suggest that sex chromosome divergence occurred contemporaneously in multiple independent lineages. This temporal clustering around 10 Mya coincides with the Middle Miocene Climatic Transition (MMCT), a period of pronounced global cooling, aridification, and habitat fragmentation that profoundly affected terrestrial ecosystems worldwide (*48*). This paleoclimatic event has been associated with biogeographic reorganizations in numerous vertebrate lineages (*49*–*51*), raising the possibility that environmental changes during this period may have created selective pressures that favored transitions from TSD to GSD.

To evaluate the broader generality of this pattern, we conducted a meta survey of 32 independent estimates of sex chromosome origins across amniotes, including 11 robust estimates in this study and 21 published estimates (table S19 and fig. S24). This dataset highlights a key limitation of currently available data: Because fully resolved X–Y (or Z–W) sequences and clearly defined evolutionary strata remain

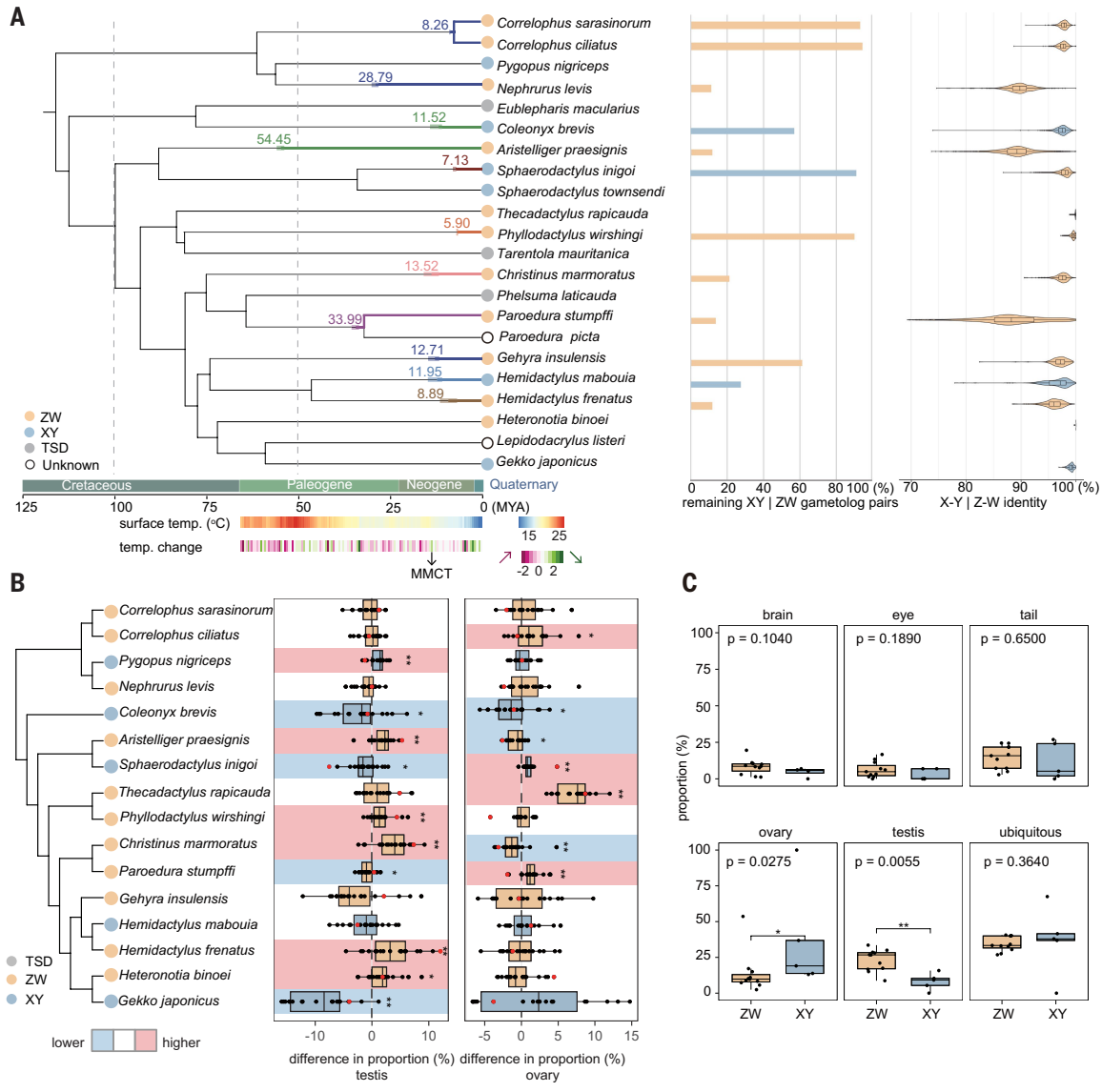

Fig. 3. Gecko sex chromosome divergence and evolution. (**A**) Gecko divergence times and estimated sex chromosome ages. Bars are colored by GACs that evolved into sex chromosomes (Fig. 2); translucent regions indicate 95% confidence intervals. Historical temperature data (*88*) are shown as average changes per 1.5 Myr (pink, warming; green, cooling). The middle panel shows the surviving gametolog pairs percentage, and the right panel shows X/Y (or Z/W) sequence identity distribution. (**B**) Proportion of testis (left) and ovary (right) preferentially expressed genes on the oldest X/Z-SDR relative to genomic background. Red dots indicate the focal gecko, while black dots indicate the ancestral states indicative by the difference in the other geckos. Background colors denote significant higher (red), lower (blue), or no difference (white) proportions. Wilcoxon rank sum test $^{**}P < 0.01$ and $^{*}P < 0.05$. (**C**) Proportions of ancestral genes changing into tissue-preferential expression in XY and ZW species. XY species show more genes evolving into ovary-biased expression, whereas ZW species show more genes evolving into testis-biased expression. No significant differences were observed for genes evolving bias in other tissues or ubiquitous expression. Wilcoxon rank sum test $^{**}P < 0.01$ and $^{*}P < 0.05$.

unavailable for many systems, robust temporal inference is restricted to only four lineages outside of geckos (table S19 and fig. S24). Within these constraints, we observed that sex chromosomes in amniotes appear nonrandomly distributed in time, with concentrations in an early Cretaceous interval previously noted (*52*) as well as around ~30 Mya involving *Siebenrockiella*, *Saltuarius*, *Paroedura stumpffi*, *Nephrurus levis*, and *Furcifer* (table S19 and fig. S24). However, given the limited sample size, uncertainties in molecular dating, regional climatic heterogeneity, and lineage-specific evolutionary trajectories, we cannot formally test for synchrony or draw definitive conclusions about universal patterns. These preliminary observations nonetheless provide a foundation for future comparative analyses as genomic resources expand and suggest that the temporal clustering observed in geckos may represent a testable framework for understanding sex-determination evolution across amniotes (*11*, *15*).

We next sought to examine the molecular mechanisms underlying the subsequent evolution of sex chromosomes. According to evolutionary theories, sex chromosomes can evolve through two primary mechanisms: successive stepwise recombination suppression that creates discrete "evolutionary strata" (*53*), or gradual progression of sequence divergence across the chromosome (*2*). Our analysis revealed clear evolutionary strata in seven gecko species, while five species exhibited gradual differentiation patterns (figs. S25 to S28 and table S17). Genomic rearrangements are usually believed to be one of the driving forces of strata formation, which can initiate recombination suppression between sex chromosomes (*11*, *13*). We observed a prominent

asymmetry in rearrangement patterns: Synteny was well preserved between X (or Z) chromosomes and their autosomal homologs, indicating that most strata-associated rearrangements occurred specifically on the Y and W chromosomes (Fig. 2A). We only found X-linked inversions in *Pygopus nigriceps* and Z-linked inversions in *Aristelliger praesignis* (Fig. 2A). This pattern mirrors observations in mammals, where Y-specific rearrangements predominate (*53*, *54*), but contrasts with birds, where both Z and W chromosomes undergo rearrangements (*55*). Most rearrangements between X and Y, or between Z and W, are associated with strata, with rearrangements found near the stratum boundary (fig. S25), consistent with their role in recombination suppression. Among gecko rearrangements, inversions were overwhelmingly prevalent, comprising 75 and 73% of rearrangements on X/Z and Y/W chromosomes, respectively, highlighting their fundamental role in establishing recombination suppression (Fig. 2A). We also identified cases of complex sex chromosome architecture, including multiple sex chromosome systems arising through independent fusion events in *Sphaerodactylus inigoi* (XX/XY1Y2) and *Coleonyx brevis* (X1X1X2X2/X1X2Y) (Fig. 2A and supplementary text). These genomic rearrangements and subsequent gene degeneration on Y/W chromosomes likely create evolutionarily stable "traps" that maintain GSD systems without reversal to ESD or turnover to different sex chromosomes.

The rate of sex chromosome divergence is fundamentally influenced by sex-specific mutation patterns. The "faster male" hypothesis proposes that males accumulate mutations at higher rates than females owing to increased cell divisions during spermatogenesis (*56*, *57*). In vertebrates with high male mutation bias, the accumulation of mutations on the sex chromosomes, particularly on the Y or W, leads to rapid divergence and, in many cases, to progressive degeneration of the nonrecombinant chromosomes (*16*). Our analysis showed that geckos exhibited a relatively low male mutation bias (0.74 to 1.51) compared with mammals (2.0 to 2.6) and birds (2.5 to 4.1) (*58*) (table S18). The lower male mutation bias in geckos may slow the process of the sex chromosome differentiation and degeneration. This reduced mutation bias may explain the observation that geckos maintain relatively homomorphic sex chromosomes (*20*) and low sexual dimorphism over long evolutionary timescales (*59*–*66*). Although we observed that XY geckos tend to have higher male mutation bias than ZW geckos (Wilcoxon rank sum test, $P = 0.0112$; fig. S29), this likely reflects estimation challenges or lineage-specific variation in mutation processes between sexes rather than systematic biological differences between sex chromosome systems (*57*).

Ancestral gene content is associated with the direction of sex chromosome evolution

The multiple independent transitions into GSD systems in geckos show that certain ancestral chromosomes tend to evolve into XY while others preferentially give rise to ZW (Fig. 2B). For example, all five geckos that recruited GAC16 as the sex chromosome evolved into ZW systems, whereas both *Sphaerodactylus* recruited GAC2 into XY systems. This nonrandom pattern raises a fundamental question about whether the ancestral gene contents of autosomal region predispose them toward specific sex-determination system.

To answer this question, we conducted a comparative transcriptomic analysis, focusing on genes within the oldest SDR, which best preserve the ancestral genomic context that existed before sex chromosome establishment. We excluded genes from younger SDR, as their expression patterns may have been confounded by sex-specific selective pressures that emerged after the establishment of XY or ZW (*67*–*69*). We categorized genes by tissue expression preference (methods) and compared the proportion of genes with tissue bias between the oldest SDR and the genomic background (i.e., autosomes and PAR). Compared with the genomic background, Z consistently harbored a higher proportion of testis preferentially expressed genes (TPEGs), whereas X showed a tendency toward lower TPEG proportion (Fig. 3B). This observation suggested two potential explanations: Either these differences represented preexisting ancestral states that influenced sex chromosome evolution, or genes underwent masculinization or feminization after sex chromosome formation.

To distinguish between these possibilities, we examined the expression profile of the orthologous genes in other gecko species where these regions remain autosomal, thereby serving as proxies for the ancestral state. This analysis revealed that 10 of the oldest regions exhibited significantly different TPEG proportions compared with the genomic background in other geckos, indicating that these regions already had biased gene content before evolving into sex chromosomes (Wilcoxon rank sum test $P < 0.05$; Fig. 3B and table S20). Among regions that showed significantly higher TPEG proportions than genomic background, 83.33% (5 of 6) evolved into the ZW system (Fig. 3B, orange boxes with red background). Conversely, among regions with significantly lower TPEG proportions, 75.00% (three of four) evolved into the XY system (Fig. 3B, blue boxes with blue background). Although the association does not reach statistical significance (Yates-corrected chi-square test $P = 0.236$), probably because of the limited sample size, the trend motivated us to assess whether the association generalizes across vertebrates more broadly.

We extended our analysis beyond geckos to examine 39 species across major vertebrate lineages, representing 15 independent amniote sex chromosome origins and 24 anamniote origins (fig. S30 & table S21). Across multiple amniote lineages with independently evolved sex chromosomes, we observed consistent patterns. In species with XY systems, including therian mammals (human), monotremes (platypus), snake (*Eryx tataricus*), and turtle (*Staurotypus triporcatus*), their SDRs consistently showed significantly lower ancestral TPEG proportions compared with genomic background (Wilcoxon rank sum test $P < 0.05$; fig. S30). Conversely, in birds (chicken) with a ZW system, its oldest SDR showed elevated ancestral TPEG proportion, although this difference did not reach statistical significance ($P = 0.1232$; fig. S30). Quantitatively, this pattern is present across all amniotes analyzed, with five of six regions with significantly higher TPEGs evolving into ZW systems, and seven of eight regions with significantly lower TPEGs evolving into XY (Yates-corrected chi-square test $P = 0.0353$). In contrast, this associative relationship was weaker or absent in amphibians and fishes (fig. S30). Among 16 frog species examined, ancestral SDRs showed no consistent correspondence between TPEG content and sex chromosome type, with both XY and ZW systems evolved from regions with either elevated or reduced ancestral TPEG proportions. In fishes, we detected no ancestral SDRs with significantly altered TPEG proportions relative to genomic background. These taxonomic differences suggest that the predictive power of ancestral gene expression may be specific to amniotes rather than universal across vertebrates. One possible explanation could be that amniotes use internal fertilization (*70*, *71*), which may intensify postcopulatory sexual selection and may amplify the evolutionary significance of testis-expressed gene content. By contrast, amphibians and fishes typically reproduce through external fertilization with massive gamete production (*70*, *71*), potentially relaxing such selective constraints.

While our analysis focused primarily on testis-biased genes, we also examined expression patterns in other tissues including ovary. Regions with biased expression in ovary and nongonadal tissues appeared to evolve randomly into either XY or ZW systems, lacking the predictive power observed for testis-biased genes (Fig. 3B, fig. S31, and table S20). This specificity may reflect the distinct evolutionary pressures associated with testis function, which represents one of the most rapidly evolving tissue types owing to intense sexual selection and sperm competition (*72*, *73*).

Our findings also revealed evidence for secondary evolutionary processes after sex chromosome establishment. Many species exhibited even more extreme TPEG proportions in their current SDRs compared with the orthologous regions in other species (Fig. 3C), indicating post-establishment masculinization or feminization. When we compared expression changes between XY and ZW species, we found that

Z chromosomes showed significantly higher rates of genes evolving into testis-preferential expression, whereas X chromosomes showed higher rates of genes evolving into ovary-preferential expression. Notably, genes changing expression preference into somatic tissues showed no significant differences between XY and ZW systems (Fig. 3C), suggesting that the observed evolutionary bias in SDR is specific to gonadal rather than general tissue-expression evolution.

It is important to note that ancestral gene content does not deterministically control sex chromosome fate. Six regions in our dataset showed unbiased TPEG content compared with the background yet still evolved into sex chromosomes (four XY and two ZW) (Fig. 3B), indicating that other factors also influence this evolutionary process. Nevertheless, this work provides the systematic empirical evidence that ancestral genomic context is associated with the direction of sex chromosome evolution and offers a potential explanation for the nonrandom patterns of sex-determination systems (i.e., XY or ZW) observed across diverse taxa.

An age-dependent gene retention mechanism in Y/W chromosomes

The evolutionary trajectory of sex chromosomes is marked by recombination suppression and progressive degeneration of the heterogametic chromosome (*16*), yet the mechanisms governing gene survival during this process remain incompletely understood. Our comparison of gene numbers between sex chromosomes and their ancestral autosomal counterparts revealed variability in degeneration extent across gecko species (Fig. 3A). This variation follows expected temporal patterns, with gene loss occurring rapidly during early evolutionary stages followed by a marked deceleration over time (fig. S32), consistent with theoretical predictions (*74*) and empirical studies in other vertebrate lineages (*55*, *75*).

To understand the evolutionary forces governing gene survival, we investigated two primary hypotheses that have emerged from studies of ancient sex chromosome systems in mammals, birds, and snakes (*42*, *54*). The first proposes that genes beneficial to the heterogametic sex are preferentially retained and potentially amplified. The second suggests that dosage-sensitive genes essential for maintaining balanced expression between sexes are protected from degeneration. Despite the diverse genomic backgrounds from which these sex chromosomes evolved, surviving genes consistently showed enrichment for fundamental cellular functions, such as nucleic acid binding and heterocyclic compound binding, but lack of enrichment for gonadal development or other sex-specific roles (table S22). This pattern supports dosage sensitivity, rather than sex-specific utility, as the primary driver of gene retention in geckos. This finding aligns with patterns seen in birds and snakes, suggesting a shared evolutionary strategy across sauropsids that contrasts with the mixed retention strategies in mammals (*54*, *76*).

To further dissect the mechanisms underlying gene retention, we conducted a detailed comparative analysis of genes on the X/Z that had retained their Y/W gametologs versus those that had lost them. We examined three key features previously associated with gene retention, haploinsufficiency (HI), expression breadth, and evolutionary constraint (dN/dS, the ratio of nonsynonymous to synonymous substitution rates) (*54*, *76*) (table S23). In species with more diverged sex chromosomes, particularly *Aristelliger praesignis* and *Paroedura stumpffi*, genes with retained gametolog pairs show significantly higher HI score and broader expression patterns, along with lower dN/dS than genes without gametolog pairs (Fig. 4A and table S23). This pattern mirrors observations from ancient sex chromosome systems in mammals, birds, and caenophidian snakes (*54*, *76*), suggesting convergent evolution toward similar retention mechanisms despite independent origins. However, *Nephrurus levis* exhibited an intermediate pattern, with significant differences in HI score and expression breadth but not dN/dS (Fig. 4A and table S23), indicating that the relative importance of these factors may shift during sex chromosome evolution. Most gecko species with younger sex chromosomes showed no significant differences between genes with retained gametolog pairs and those that without gametolog pairs across these features. Only a few exceptions appeared in *Sphaerodactylus inigoi*, and *Phyllodactylus wirshingi* differed in expression breadth, whereas *H. mabouia* differed in HI score (Fig. 4A and table S23). This age-dependent variation in retention mechanisms suggests that the early stages of sex chromosome differentiation may be governed by different selective pressures or that insufficient time has elapsed for strong selection to act on gene retention. This observation suggests that the retention mechanisms may not be uniform across all stages of sex chromosome evolution and highlights the importance of studying systems at various evolutionary stages.

Even among gecko species with similar estimated ages (7 million to 13 million years), Y/W gene retention rates varied widely, from 20 to 97% (Fig. 3A). This variation cannot be explained by differences in HI, expression breadth, or evolutionary constraint, as we found no significant correlations between these factors and retention rates (fig. S33; $P > 0.05$). The fact that these species have nonhomologous SDRs suggests that ancestral gene content may influence retention patterns (*77*, *78*), although our current analysis could not definitively establish this relationship. Other influences, such as differences in effective population size, recombination landscape, mating system, or lineage-specific biological constraints, potentially affect retention patterns in ways not captured by our current analytical framework (*79*). The nonhomologous nature of SDRs across gecko species means that each system began with different ancestral gene complements, potentially creating distinct evolutionary trajectories even under similar selective regimes. Future work incorporating population genomic data and expanded phylogenetic sampling will be necessary to disentangle these factors' relative contributions.

Diverse degree of dosage balance with consistent heterogametic up-regulation

The degeneration of sex chromosome creates a fundamental genomic imbalance between sexes, as the heterogametic sex carries only one functional copy of genes that exist in two copies in the homogametic sex (*12*, *80*, *81*). This loss of genes on the Y/W can lead to dosage imbalance, potentially leading to deleterious effects to organism fitness (*82*). While diverse taxa vary in both the extent and mechanism of dosage balance (DB) and dosage compensation (DC), the underlying principles governing this diversity are not yet fully understood (*80*).

We examined DB between sexes across 12 GSD gecko species with fully resolved SDRs. By comparing expression levels of sex-linked genes between the homogametic and heterogametic sex across multiple somatic tissues, we found that most geckos exhibit partial DB, with X:XX (or Z:ZZ) expression ratios typically between 0.5 and 1 (Fig. 4B and table S24). However, three geckos (*Christinus marmoratus*, *Hemidactylus mabouia*, and *Sphaerodactylus inigoi*) appear to exhibit full DB with similar expression levels between sexes (Fig. 4B and table S24). The degree of DB varied substantially among species but showed no correlation with sex chromosome divergence level (fig. S34; $P > 0.05$), nor did it differ between XY and ZW systems (Fig. 4B). This pattern persisted even among species sharing homologous SDRs such as *Aristelliger praesignis* (ZW) and *Coleonyx brevis* (XY), which display similar levels of DB despite evolving opposite heterogametic systems (fig. S35). Together, these results suggest that the degree of DB may be shaped by lineage-specific or gene-level properties rather than by sex chromosome system or age.

To further explore the variation among genes, we classified X/Z genes into genes with retained gametolog pairs and genes without gametolog pairs. Across species, genes without gametolog pairs generally exhibited stronger DB between sexes than those with retained gametolog pairs (Fig. 4B and fig. S36; paired Wilcoxon signed-rank test $P < 0.05$). When expression of the Y/W gametolog was included, genes with retained gametolog pairs in the heterogametic sex (i.e., X/Z+Y/W) reached expression levels comparable to the homogametic sex and exhibited

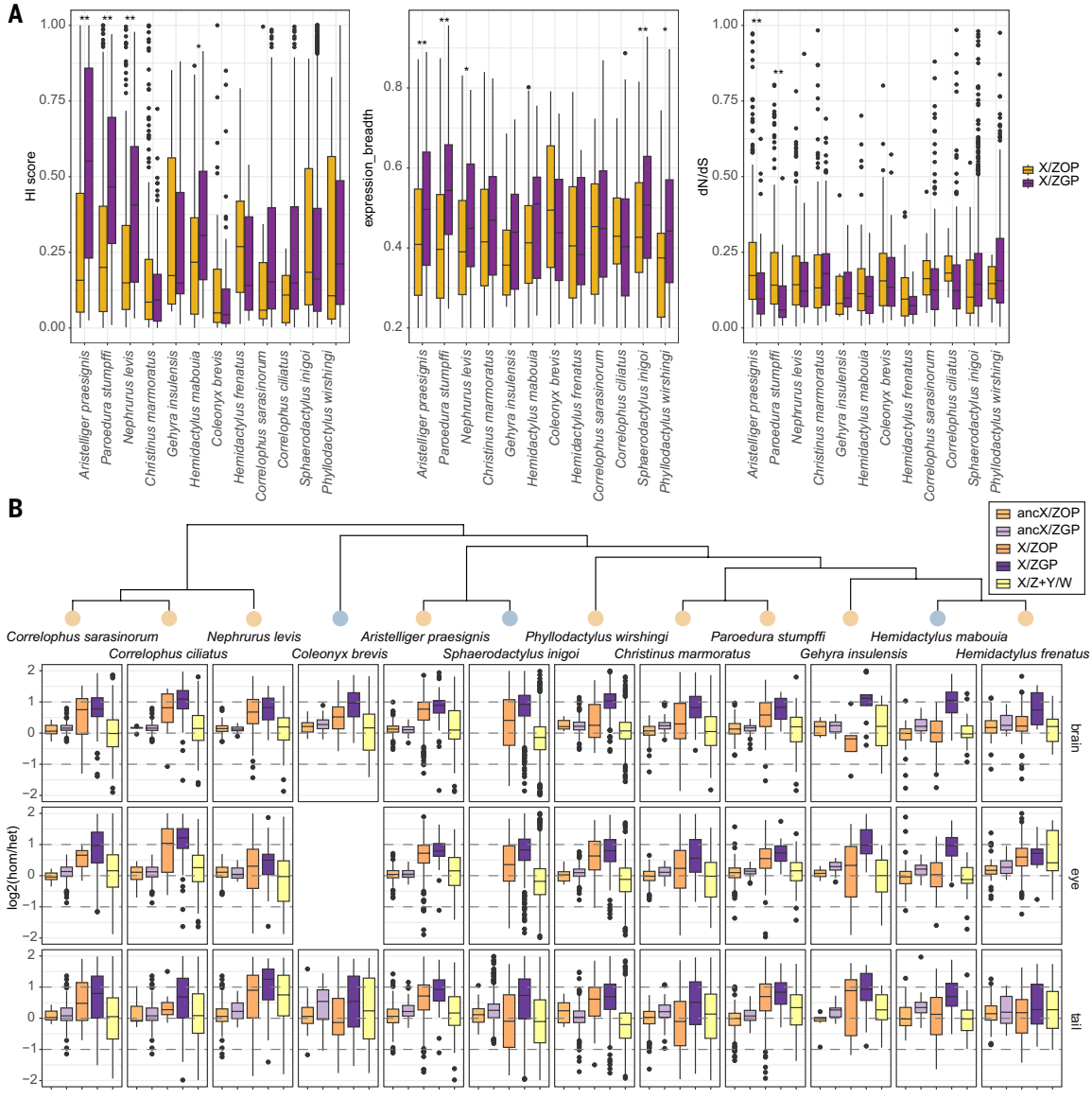

Fig. 4. Examination of the factors of Y/W chromosome gene survival in geckos. (**A**) Comparison of HI score, expression breadth, and dN/dS between X/Z genes with gametolog pairs (X/ZGP) and X/Z genes without gametolog pairs (X/ZOP). Unlike the observations in humans and birds, only a few geckos exhibited significant differences between XZ/GP and X/ZOP. Species are ordered by sex chromosome age. $^{**}P < 0.01$, $^{*}P < 0.05$. (**B**) The pattern of dosage balance in geckos. Comparison of the ratio of expression level between homogametic (hom) sex and heterogametic (het) sex in the somatic tissues (brain, eye, and tail) of the geckos. For *Sphaerodactylus inigoi*, the expression ratio pattern of left head and right head are shown on the brain and eye row, respectively. Log_2(hom/het) of zero indicates complete dosage compensation, and log_2(hom/het) of one indicates no dosage compensation.

stronger DB compared with genes without gametolog pairs (Fig. 4B and fig. S36; paired Wilcoxon signed-rank test $P < 0.05$). This pattern is consistent with a buffering model in which gene regulatory networks partially compensate for reduced copy number when one homolog is missing but achieve near-normal expression when both copies are present (*82*). This gene-by-gene variation in dosage balance based on gametolog availability further supports the role of intrinsic network resilience in responding to dosage imbalances, with factors such as chromatin accessibility potentially mediating this regulatory flexibility (*83*).

To determine the regulatory direction underlying DB, we distinguished DC from DB (*80*) by comparing current to ancestral sex-linked gene levels inferred from orthologous autosomal genes in related species. This phylogenetic approach revealed that DC in geckos operates through up-regulation in the heterogametic sex rather than down-regulation in the homogametic sex. Expression level in the homogametic sex remained similar to the ancestral autosomal state, while the heterogametic sex showed elevated expression of sex-linked genes, with compensation ratios generally exceeding 0.5-fold of the expected dosage difference (fig. S37 and table S24). This pattern was observed irrespective of whether species displayed partial or full DB and parallels the mode of regulation reported in birds and snakes (*13*, *84*). This suggests that partial up-regulation in the heterogametic sex represents an evolutionarily common regulatory response that can persist across diverse taxa without necessarily evolving into complete chromosome-wide compensation.

Discussion

Our comparative genomic analysis of 19 gecko species provides insights into the evolutionary dynamics of sex chromosome systems.

Gecko sex chromosomes evolved independently from 16 different ancestral chromosomes, with 5 of 11 datable origins coinciding near the MMCT (~10 Mya). The nonrandom temporal distribution of gecko GSD origins, supported by Monte Carlo simulation, coincides with MMCT and raises the possibility of an association between large-scale environmental change and concordant shifts in sex-determination systems. This pattern suggests that shared environmental context may influence the concordant timing of sex chromosome divergence across multiple independently evolving gecko lineages. By increasing environmental variability and reducing the reliability of temperature cues, such events may have favored GSD as a mechanism for stabilizing sex ratios (*1*, *85*, *86*).

Our analyses also showed that ancestral gene expression is associated with evolutionary trajectories, with testis-enriched genomic regions preferentially evolving into ZW systems, whereas testis-depleted regions favor XY evolution. The mechanistic basis for this pattern likely reflects evolutionary optimization of sex-biased gene expression during the transition to genetic sex determination. Testis-enriched regions may be preferentially recruited into ZW systems because subsequent W chromosome degeneration minimally affects male-specific functions, while the Z chromosome can maintain essential male-biased expression patterns. Conversely, testis-depleted regions more readily evolve into XY systems because Y chromosome degeneration has more consequences for male reproductive function. The extension of this pattern to mammals, some snakes, and turtles indicates that genomic predisposition may represent a contributing factor in the direction of sex chromosome evolution across amniotes. Nevertheless, we acknowledge that the test remains statistically limited ($n = 14$), and broader datasets will be required to test its robustness under formal clade-balanced sampling.

On the basis of these findings, we propose a unified evolutionary model that integrates environmental pressures, genomic predisposition, and evolutionary constraints into a coherent framework for understanding sex chromosome evolution (Fig. 5) composed of four phases. In phase 1 ("environmental destabilization"), major climatic transitions increase environmental variance and unpredictability, creating selective pressures that destabilize TSD and favor the evolution of GSD. In phase 2 ("biased genomic recruitment"), the transition to GSD is not random but rather reflects genomic predisposition, with testis-enriched chromosomal regions preferentially recruited into ZW and testis-depleted regions into XY, optimizing the maintenance of sex-biased gene expression. In phase 3 ("progressive differentiation"), after recruitment, sex chromosomes undergo differentiation through recombination suppression, creating either discrete evolutionary strata or gradual differentiation patterns. And in phase 4 ("evolutionary stabilization"), progressive Y/W chromosome degeneration, coupled with the evolution of dosage compensation mechanisms, creates evolutionary constraints that stabilize sex chromosome systems and prevent turnover. This framework moves beyond descriptive accounts to relate environmental context and ancestral gene content. It suggests that sex chromosome diversity across vertebrates may reflect nonrandom responses to environmental pressures operating within the constraints of genomic architecture. The model's integration of multiple evolutionary scales—from environmental triggers to molecular mechanisms—provides an improved understanding of how fundamental genomic innovations arise and persist in natural populations.

Several areas warrant future investigation. Assembly challenges in highly homomorphic sex chromosome pairs limited our ability to fully resolve their structure and estimate sex chromosome ages in a subset of species, and advances in long-read sequencing will help overcome these constraints. Establishing direct mechanistic links between paleoclimatic events and sex-determination transitions will require additional independently datable GSD origins and complementary lineage-specific paleoclimate content. Sex-determination systems remain unknown for the majority of gecko species (*18*, *19*), and broader taxonomic sampling may reveal additional systems that could refine the temporal distribution pattern and evaluate the generality of patterns reported here. Although our analyses suggest a possible association

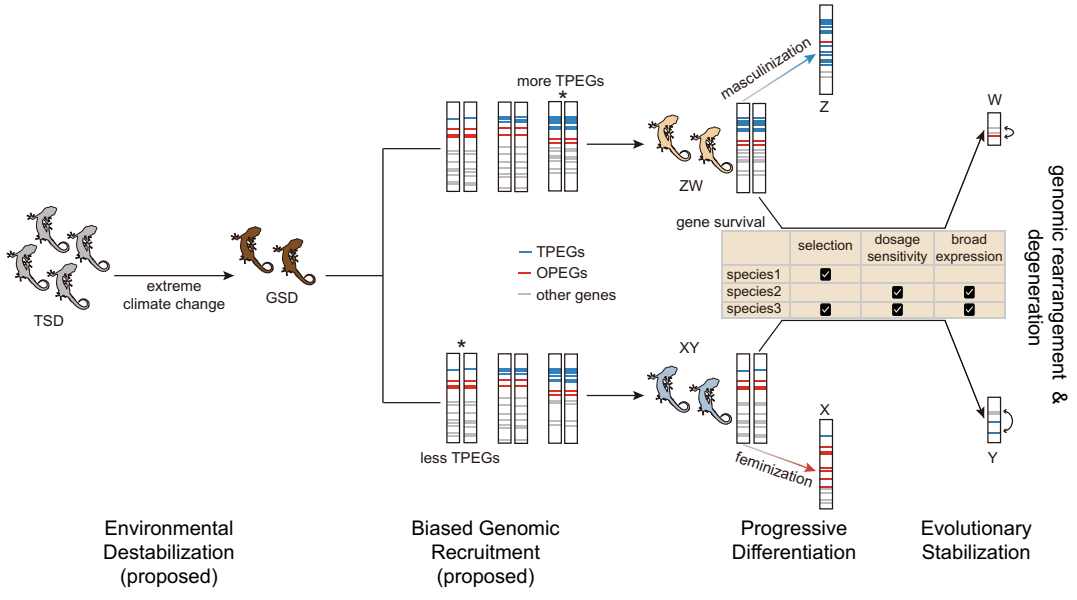

Fig. 5. Model of sex chromosome evolution. Ancestral amniotes are hypothesized to have environmental sex determination, for example, TSD (*17*,*18*). Environmental instability may trigger the transition from TSD to a GSD system. The proportion of TPEGs influence whether the XY or ZW system will evolve. Specifically, the region or chromosome with a higher proportion of TPEGs than the genomic background tends to evolve into the ZW system, whereas the region or chromosome with a lower proportion of TPEGs than the genomic background tends to evolve into the XY system. After the establishment of the GSD system, the Z chromosome would further masculinize by evolving more genes preferentially expressed in testis, while the X chromosome is feminized by evolving more genes preferentially expressed in ovary. Despite the degeneration of the Y/W chromosome, some genes may still survive on the chromosome. However, the survival of Y/W chromosome genes in different species might be affected by their difference in sex chromosome age and different selection forces. [Gecko silhouette is copyrighted by Stuart V. Nielsen]

between ancestral TPEG abundance and the evolution of XY versus ZW, the underlying regulatory mechanisms warrant deeper investigation. The absence of canonical vertebrate sex-determining genes from some gecko SDRs (supplementary text and table S25), despite their presence at autosomal locations, suggests that sex-determination pathways from geckos may use novel molecular mechanisms, and our preliminary candidate genes provide important starting points for functional validation.

Materials and methods are available in the supplementary materials.

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ACKNOWLEDGMENTS

We thank P. Doughty for assistance with fieldwork and permitting. We also gratefully acknowledge the support of the K.C. Wong Education Foundation. We also acknowledge the Information Technology Center of Zhejiang University and China Mobile Zhejiang Co. Ltd. (Hangzhou Branch) for providing the computation resource. Genome assembly was performed in association with the DNA Zoo Consortium (www.dnazoo.org), which acknowledges support from Illumina, IBM, and the Pawsey Supercomputing Center. **Funding:** This work was supported by National Natural Science Foundation of China (grant 32388102) to G.Z. and P.S., Shenzhen Science and Technology Program (grant RCBS20231211090513005) and the Young Elite Scientists Sponsorship Program by CAST (2023QNRC001) to Y.Z., Fundamental and Interdisciplinary Disciplines Breakthrough Plan of the Ministry of Education of China (JYB2025XDXM508), NSF-DEB 1657662 to T.G., and the New Cornerstone Science Foundation through the XPLORER Prize and K.C. Wong Education Foundation and Zhejiang University Global Partnership Fund to G.Z. E.L.A. was supported by the Welch Foundation (Q-1866), an NIH Encyclopedia of DNA Elements Mapping Center Award (UM1HG009375), a US-Israel Binational Science Foundation Award (2019276), the Behavioral Plasticity Research Institute (NSF DBI-2021795), and an NSF Physics Frontiers Center Award (NSF PHY-2019745). **Author contributions:** Conceptualization: G.Z., T.G., P.S., Y.Z.; Resources: T.G., B.J.P., X.G., Y.L., S.V.N., A.H.G., T.E., A.G., I.B.; Data generation: Zhe.S., O.D., Z.Co., R.K., A.D.O., L.J., Zha.S.; Formal analysis: Y.Z., J.J., C.J., X.L., Zhe.S., L.H., W.C., B.J.P., O.D., D.W., E.L.A., Y.X., Y.Q., Z.Co., Z.Ca., Y.P., Z.Z., Q.Z.; Writing – original draft: Y.Z., J.J.; Writing – review & editing: G.Z., Y.Z., T.G., B.J.P., A.H.G.; Visualization: Y.Z., J.J., C.J., W.C., A.H.G.; Supervision: G.Z., Y.Z., E.L.A.; Funding acquisition: P.S., G.Z., T.G., Y.Z., E.L.A. **Competing interests:** E.L.A. and O.D. hold patents related to Hi-C. E.L.A. is on the scientific advisory board of Colossal Biosciences. **Data, code, and materials availability:** The gecko genome and transcriptome data have been deposited at GenBank (project accession PRJNA1288340) and CNGBdb (accession CNP0005488). Hi-C data are available at NCBI under PRJNA512907 (accession numbers SRR35123232–SRR35123304). Public databases used in this study include NCBI (https://www.ncbi.nlm.nih.gov/), UniProt (https://www.uniprot.org/), and Repbase (https://www.girinst.org/repbase/). Important data generated by this study are available at Figshare (https://figshare.com/s/31833ea74c36f3f36fdd). In-house scripts used in this study are available on GitHub (https://github.com/Dived-Jin/Gecko_Sexchromosome) and Zenodo (*87*). No new materials were generated for this study.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.aec3449
Materials and Methods; Supplementary Text; Figs. S1 to S37; Tables S1 to S25; References (*89*–*177*); MDAR Reproducibility Checklist

Submitted 16 September 2025; resubmitted 15 March 2026; accepted 24 June 2026
10.1126/science.aec3449

Genomic predisposition is associated with the direction of sex chromosome evolution

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Science **393** (6814), eaec3449. DOI: 10.1126/science.aec3449

Editor's summary

Despite their importance in reproduction, sex chromosomes have often shown signs of rapid evolution. Zhou *et al*. sequenced the genomes and examined the sex chromosomes of 19 gecko species, which have evolved temperature-dependent as well as XY and ZW genetic sex determination systems across lineages. Although some ancestral chromosomes evolved into sex chromosomes in multiple species, these often did not stem from homologous regions. When the authors examined the gene content of these regions, they found gene clusters with sex-biased gene expression, even in species with different sex determination systems. Given the prevalence of recombination suppression locking genes together on sex chromosomes, such properties may tip evolution toward XY or ZW systems. —Corinne Simonti